

**REMARKS**

Applicant respectfully requests reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

Claims 1-6, 8-15 and 23-27 are pending in the application. Claims 1, 2, 9, 12-15, and 23-27 are requested to be cancelled. Claim 3 has been amended into independent format as the claim from which it originally depended has been cancelled. The amendment to claim 3 is supported by the claim as originally filed.

This amendment changes and deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier. After amending the claims as set forth above, claims 3-6, 10 and 11 are now pending in this application.

Following the amendments and in light of the arguments below, applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

**I. Claim Rejections based on 35 U.S.C. § 112, second paragraph**

Applicants thank the Examiner for the withdrawal of the rejection of claims 3-6, 8 and 10-11 under 35 U.S.C. § 112, second paragraph.

**II. Claim Rejections based on 35 U.S.C. § 101**

The Examiner continues to reject claims 3-6, 10 and 11 under 35 U.S.C. § 101 because the “claimed invention is not supported by either specific and substantial asserted utility or well established utility.” This rejection was argued in the response to the previous Office Action but the Examiner found the arguments unpersuasive. Respectfully, based on the reasons set forth below, Applicants traverse and continue to assert that the currently pending claims are in condition for allowance.

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The Examiner states that

[T]here is no disease or disorder correlated with the leukocyte and blood associated protein (LBAP) encoded by the nucleic acid sequence of the invention. ... Leukocyte and blood associated protein (LBAP) encoded by novel leukocyte and blood associated polynucleotide does not appear to have structural similarity to haemopoietic membrane proteins, but even if they did demonstrate a level of structural homology, since the function of leukocyte and blood associated protein (LBAP) is not known it would not be conclusive to assume, solely based on structure homology, that they have the same function and have the same utility.

First, Applicants respectfully disagree with the Examiner's statement that that LBAP does not appear to have structural similarity to haemopoietic membrane proteins. As supported in the specification and submitted in the response of April 30, 2004, LBAP-5 shares 77% homology over 132 amino acid residues with JB542. In the area believed to be the transmembrane region of JB542, LBAP-5 shares 88% homology over 50 amino acid residues. Baird *et al.*, J Biol Chem 276(12): 9189-9198 (2001) (Exhibit A); Sequence Alignment, LABP-5 vs JB542 by BLAST analysis (previously presented). Further, as demonstrated by BLAST alignment, LBAP-5 shares transmembrane domain sequence homology with other leukocytes and blood associated proteins. For example, LBAP-5 demonstrates approximately 55% homology with the transmembrane domain of IFITM1 (Leu-13), an interferon-induced member of the haemopoietic membrane protein family. Sequence Alignment, LABP-5 vs IFITM1 by BLAST analysis (Exhibit B); the putative transmembrane domain is underlined. Overall, the complete homology between LBAP-5 and IFITM1 is 51% over 72 amino acids. *Id.* Although protein sequence comparisons as indicators of comparative functions are never completely conclusive, Applicants disagree with the Examiner that comparisons based on sequence homology are inadequate to show utility.

It is well-known in the art that high levels of sequence identity are highly predictive of function. For example, scientific literature exists supporting the assertion that sequence identity between proteins suggests similar function. As submitted in the previous response, Brenner *et al.* disclose quantitative criteria for evaluating whether structural similarity supports a conclusion that two proteins are homologous. Brenner *et al.*, Proc. Natl. Acad. Sci. 95: 6073-6078 (1998) (previously presented). Brenner *et al.* demonstrate that 40% sequence identity over at least

70 residues, is a reasonable and reliable threshold for signifying homology. Brenner *et al.*, page 6076, third paragraph, right-hand column. In the present case, the sequence identity between LBAP-5 and JB542 is 77% over 132 amino acid residues and the sequence identity LBAP-5 and IFITM1 is 51% over 72 amino acid residues.

Additionally, Bork teaches that it is routine in the art to use sequence comparisons to predict protein function. Bork discloses a 70% accuracy rate in bioinformatics-based predictions. Bork, *Genome Research* 10(4): 398-400 (2000), at page 400, first column (Exhibit C). Furthermore, for predicting “functional features by homology,” Table 1 of Bork discloses a 90% accuracy rate. Thus, Bork corroborates the use of reasonable quantitative thresholds, such as those taught by Brenner *et al.*, and provides further support that one of skill in the art would consider LBAP to be a leukocyte or blood associated protein.

In light of the high correlation between sequence identity and functional similarity, one of skill in the art using the specification disclosure identifying LBAP-5 as a putative haemopoietic membrane protein with sequence similarity to other functional haemopoietic membrane proteins would recognize LBAP-5 as a haemopoietic membrane protein with known functions. The identity with IFITM1, an interferon-induced haemopoietic membrane protein with known utility, is highly indicative of the function of LBAP-5.

IFITM1, which as stated above shares greater than 50% sequence homology to LBAP-5, is a receptor molecule important in the regulation of L-selectin expression. Frey *et al.*, *J Immunol* 158(11): 5424-5434 (1997) (Exhibit D). At the time of filing, L-selectin expression had been linked to many different pathologies including chronic inflammatory responses, autoimmune disorders, and metastatic dissemination in leukemia or lymphoma. Because IFITM1 regulates L-selectin, which is known to be linked to many different pathologies, use of IFITM1 to control L-selectin expression clearly demonstrates appreciable utility. Further, regulation of IFITM1 by monoclonal antibodies inhibits the proliferation of leukemic B cells, suggesting the use of IFITM1 as a potential target for leukemia suppression. Evans *et al.*, *Blood* 12: 2583-2593 (1990) (Exhibit E).

One of skill in the art would understand that based on the instant specification and sequence homology to IFITM1, there is a substantial likelihood that LBAP-5 demonstrates utility

similar to the utility of IFITM1, such as a potential target for leukemia suppression. Applicants remind the Examiner that absolute certainty of utility is not required and it is enough that the Applicants have proven a substantial likelihood of utility. *Brenner v. Manson*, 383 U.S. 519 (1966). This substantial likelihood of utility is supported by the disclosure in the specification, which states “the expression of LBAP is closely associated with cell proliferation, cancer and inflammation. Therefore LBAP appears to play a role in autoimmune/inflammatory disorders and cell proliferative disorders including cancer.” Specification, page 29, lines 9-12. The skilled artisan understanding the utility of homologous haemopoietic membrane proteins in conjunction with the specification would determine that the claimed proteins and nucleotides of the instant invention have a determined function with real world significance.

Regarding the Examiner’s specific rejections of claim 5 and claim 6 as lacking utility, because LBAP-5 demonstrates utility when claimed alone, LBAP-5 in conjunction with a promoter sequence or a transformed cell also demonstrates utility. As an example of the utility, claim 5 and claim 6 point to products that could be used for treatment of LBAP-5 related disorders.

Because claims 3-6, 10, and 11 claim a polynucleotide and polypeptide with demonstrated utility, Applicants respectfully submit that the 35 U.S.C. § 101 utility requirement has been satisfied and the 35 U.S.C. § 101 rejection should be withdrawn.

### **III. Claim Rejections based on 35 U.S.C. § 112, first paragraph**

The Office Action states in its rejection of claims 2-6, 10 and 11 under 35 U.S.C § 112, first paragraph that “since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention.” Applicants have established a substantial and well-established utility as described above. Contrary to the Examiner’s assertion, as laid out above, the specification discloses to one of skill in the art how to use the nucleic acid and protein molecules described in the pending claims. Therefore, the 35 U.S.C. § 112, first paragraph, rejection is improper and should be withdrawn.

The Examiner also rejected claims 3-6, 10 and 11 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. As argued in the response of April 30, 2004, Applicants do not believe the claims cover genomic sequence that the inventors were not in possession of at the time of filing. Claims 3-6, 10 and 11 all require that the isolated polynucleotide must encode the polypeptide clearly disclosed in the specification. This encompasses only the coding sequence for SEQ ID NO.: 5, as found, for example, in SEQ ID NO.: 10. The claims cannot therefore encompass the genomic DNA sequence which applicant does not claim to be in possession of. With respect to potential splice variants arising from alternative exon splicing, again, no claim to such splice variants, or any other variants for that matter are recited in the claim. Withdrawal of the rejection of claims 3-6, 10 and 11 under 35 U.S.C. § 112, first paragraph for lack of adequate written description is requested.

### CONCLUSION

In light of the above amendments and remarks, Applicants submit that the present application is fully in condition for allowance, and request that the Examiner withdraw the outstanding objections/rejections. Early notice to that effect is earnestly solicited. The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would result in allowance of the present application.

Respectfully submitted,

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## Differentiating Embryonal Stem Cells Are a Rich Source of Haemopoietic Gene Products and Suggest Erythroid Preconditioning of Primitive Haemopoietic Stem Cells\*

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The difficulties associated with studying molecular mechanisms important in hemopoietic stem cell (HSC) function such as the problems of purifying homogeneous stem cell populations, have prompted us to adapt the murine ES cell system as an *in vitro* model of HSC generation and function. We now report that careful analysis of the time course of HSC generation in differentiating ES cells allows them to be used as a source of known and novel hemopoietic gene products. We have generated a subtracted library using cDNA from ES cells collected just prior to and just following the emergence of HSCs. Analysis of this library shows it to be a rich source of known hemopoietic and hemopoietic related gene products with 44% of identifiable cDNAs falling into these camps. We have demonstrated the value of this system as a source of novel genes of relevance to HSC function by characterizing a novel membrane protein encoding cDNA that is preferentially expressed in primitive hemopoietic cells. Intriguingly, further analysis of the known components of the subtracted library is suggestive of erythroid preconditioning of the ES cell-derived HSC. We have used dot-blot and *in situ* analysis to indicate that this erythroid preconditioning is probably restricted to primitive but not definitive HSC.

The hemopoietic stem cell (HSC)<sup>1</sup> occupies a pivotal position within the hemopoietic hierarchy and it is at this cellular level that all hemopoietic function is ultimately regulated (1). It is also at this level that dysfunctions involved in the pathogenesis of leukemias and myeloproliferative disorders frequently arise (2). For these reasons, it is clear that a more complete understanding of the molecular mechanisms regulating the generation and function of hemopoietic stem cells is central to our appreciation of physiological and pathological stem cell function. Unfortunately, a number of practical limitations associated with studies on adult HSC have precluded in depth analyses of genes of relevance to stem cell function. Foremost among these difficulties is the problem of purifying homogeneous populations of stem cells for molecular analysis. To alleviate the problems associated with studies on adult HSC, we have recently turned our attention to the murine *in vitro* embryonal stem (ES) cell system and have been attempting to develop this as a tractable model of stem cell generation and function that may be more amenable to in depth molecular analyses.

Embryonal stem cells are totipotent cells derived from the murine blastocyst at 3.5 days post-coitum and are maintained in an undifferentiated state by *in vitro* culture in the presence of the differentiation inhibiting agent leukemia inhibitory factor (LIF) (3–5). Upon removal of the LIF, ES cells when cultured as aggregates, or embryoid bodies (EBs) will spontaneously commit *in vitro* to a range of embryological tissues including epidermis (6), neuronal and glial cells (7), muscle cells (8), and most notably hemopoietic cells (9). Hemopoiesis in differentiated EBs is typically represented by regions of hemoglobinization which are referred to as blood islands and which are composed principally of erythrocytes with small numbers of macrophages (9). This hemopoietic cell generation occurs in the absence of exogenous growth factors, however, addition of appropriate hemopoietic growth factors or use of supportive stromal cells during the differentiation process can lead to the generation of progenitor cells for all hemopoietic lineages in the developed embryoid body (10–16). This therefore suggests that at some point between being an ES cell with no direct hemopoietic potential and being a developed EB with the capacity to contain progenitors for all of the hemopoietic lineages, some of the ES cells within an EB must commit to the hemopoietic system, *i.e.* they must become hemopoietic stem cells. Indeed a number of studies has demonstrated the presence of cells at

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<sup>1</sup> The abbreviations used are: HSC, hemopoietic stem cell; ES, embryonal stem; EB, embryoid body; PCR, polymerase chain reaction; LIF, leukemia inhibitory factor.

various stages of hemopoietic development, from hemangioblasts to primitive long-term and short-term repopulating stem cells as well as committed progenitors of the myeloid and lymphoid lineages, in the developing EBs (12, 14, 16–18).

We have used a range of *in vitro* and *in vivo* assays to demonstrate a reproducible temporal pattern of emergence of long-term repopulating and short-term repopulating HSC in EBs post-LIF removal *in vitro*. Thus, while at day 3 post-initiation of differentiation no HSCs are detectable in the developing EBs, by day 4 long-term repopulating stem cells, and by day 5 short-term repopulating stem cells are detectable (19). This stem cell generation requires no exogenous growth factors and precedes the emergence of mature hemopoietic cells or blood islands. The reproducible time frame of HSC emergence in this *in vitro* system and the ease of generation of large numbers of EB-derived cells has prompted us to examine the usefulness of the day 3/day 5 time frame as a source of known and novel hemopoietic gene products. We now report the characterization of a subtracted library generated using cDNA from day 3 and day 5 embryoid bodies. Our results suggest that of the identifiable cDNAs within the subtracted library, ~46% are predominantly associated with hemopoietic or hemopoietic supportive cells confirming the usefulness of the ES cell system as a source of known hemopoietic gene products. Characterization of a novel membrane encoding cDNA from the subtracted library has further emphasized the value of this system as a source of novel genes of relevance to hemopoiesis. In addition, analysis of the hemopoietic genes identified in the subtracted library is indicative of erythroid pre-conditioning of the primitive hemopoietic stem cell.

#### EXPERIMENTAL PROCEDURES

**ES Cell Culture and Embryoid Body Formation and Differentiation**—The EFC-1 ES cell line was routinely passaged and maintained in an undifferentiated state as described previously (19, 20). EBs were generated by hanging drop culture of 10  $\mu$ l of ES cells at a concentration of  $3 \times 10^4$ /ml in the presence of LIF in a humidified, 5% CO<sub>2</sub> atmosphere. After 2 days, the EBs were harvested into a Petri dish, washed clear of the LIF, and differentiation allowed to proceed in LIF-depleted medium at a concentration of  $10^3$  EBs/10-ml culture medium. At days 3 and 5 post-differentiation initiation, EBs were harvested and the presence of HSCs determined using the CFU-A assay as previously described (19). The remaining EBs were processed for mRNA and cDNA generation as described below.

**Isolation of mRNA and Generation of cDNA from the Day 3 and Day 5 EBs**—Large scale differentiation of EBs was carried out on a number of occasions until high level CFU-A generation was achieved between days 3 and 5 post-initiation of differentiation. EBs from this experiment were harvested and mRNA produced using a Stratagene Messenger RNA isolation kit. Aliquots (5  $\mu$ g) of the mRNA samples were used to generate day 3 and day 5 full-length cDNA libraries in ZAP Express (Stratagene) according to manufacturers instructions.

**Subtractive Hybridization**—Subtractive hybridization was performed according to the method of Wang and Brown (21) with the modification of Balzer and Baumlein (22) which involves the use of alternative linkers on the driver and tracer cDNA populations thus minimizing driver cDNA carry-over which may complicate the analysis of the subtracted library. Briefly this method involved the preparation of mRNA from day 3 and day 5 EBs which was then converted to double-stranded cDNA using the Invitrogen Copy Kit. cDNA from the day 3 and day 5 EBs was then digested separately with *AluI* and with a mixture of *AluI* and *RsaI* and these two digests pooled for each time point. Both enzymes are "blunt end" cutters and have a four-base recognition site resulting in a reduction of the average size of the cDNA population to around 500 base pairs. At this stage, double-stranded linkers were generated according to the method of Balzer and Baumlein (22). The oligonucleotides used to generate these linkers were: (a) 5'-TAGTCCGAATTCAAGCAAGAGCACA-3'; (b) 5'-CTCTTGCTTGAA-TTCGGACTA-3'; (c) 5'-AGCCATTCTAGACGTGTAACGTGATA-3'; (d) 5'-AGTTACACGCTCTAGAATCGCT-3'.

Oligonucleotides a, b, c, and d were phosphorylated and ab and cd pairs allowed to anneal to form double-stranded linkers. These linkers were ligated onto either the digested day 3 EB cDNA (ab linker pairing)

or the day 5 EB-digested cDNA (cd linker pairing). These linkers serve both as PCR primer recognition site for amplification of the cDNA populations but also as restriction enzyme sites for cleaving prior to cloning into sequencing vectors. Thus, and as underlined above, the ab linker pairing is cleavable with *EcoRI* and the cd pairing with *XbaI*.

The linker ligated on day 3 and day 5 cDNA populations were separated from residual linkers and size fractionated on a 1.4% low melting point agarose gel. From this gel a slice corresponding to a size range of 200 base pairs to 2 kilobase pairs was cut out for the day 3 and day 5 cDNA populations. These low melting point-agarose cDNA preparations were used as templates for PCR amplification of the day 3 and day 5 cDNA populations using the shorter of the two primer pairs as PCR primer. Driver cDNAs were biotinylated using the Photobiotin reagent supplied by Vector Laboratories according to manufacturers instructions.

The subtractive hybridization involved a combination of short and long hybridizations and a simultaneous generation of both day 5 and day 3 sequence-enriched libraries (see Ref. 21 for a detailed discussion of the subtractive strategy). For each, biotinylated driver and nonbiotinylated tracer were mixed at a 20:1 molar ratio and co-precipitated. The mixture was then resuspended in 20  $\mu$ l of 10 mM Tris, 1 mM EDTA, pH 8, and boiled for 3 min. This was then mixed with an equal volume of  $2 \times$  hybridization buffer (1.5 M NaCl, 50 mM Hepes, 10 mM EDTA, 0.2% SDS, pH 7.5) overlaid with mineral oil and then boiled for a further 3 min to ensure denaturation. The denatured cDNA samples were then allowed to hybridize at 68 °C for 2 h (short hybridization) or 20 h (long hybridization) following which 9 volumes of 10 mM Hepes, 1 mM EDTA, pH 7.5, prewarmed to 55 °C was added and the tubes incubated at 55 °C for 5 min. The aqueous phase was then transferred to a fresh tube to which 20  $\mu$ l of streptavidin at 2  $\mu$ g/ $\mu$ l (in 0.15 M NaCl, 10 mM Hepes, 1 mM EDTA, pH 7.6) was added and the mixture incubated at room temperature for 20 min. Protein and protein-DNA complexes were removed by phenol/chloroform extraction followed by four further streptavidin incubation and extractions. Finally the subtracted material was subjected to two more phenol/chloroform extractions and one extraction with chloroform.

After 6 rounds of short and long subtractions (21), cDNA fragments were cloned into Bluescript (pSK+) and random clones selected for sequencing. Sequencing was performed on an Applied Biosystems automated sequencer.

**Northern and Southern Blotting**—For Northern blotting, 1.4% formaldehyde-agarose gels were run with 2 or 20  $\mu$ g of mRNA or total RNA, respectively, and blotted according to standard protocols onto Hybond-N. Similarly for Southern blots, agarose gels with 5  $\mu$ g of cDNA per lane were run and blotted onto Hybond-N. All probes were labeled by random priming using the Amersham Pharmacia Biotech "Ready to Go" labeling kit.

**Isolation of the Full-length cDNA for JB542**—The full-length JB542 cDNA was isolated from the day 5 EB cDNA library by PCR using two primers internal to the JB542 sequence and the flanking T3 and T7 sites in pBK-CMV which can be rescued for the Zap Express  $\lambda$  vector. The internal JB542 primers used were: 1) 5'-TTCCAGCCATCTTCT-GGTCTCGGGCC-3'; 2) 5'-GGCCCCGAGACCAGAAGATGGCTGGGAA-3'. Following sequencing of the full-length cDNA, it was re-derived from murine FDCPmix RNA by reverse transcriptase-PCR using PFU *Taq* polymerase and three independent clones were analyzed to verify sequence.

**FDCPmix Culture and Differentiation**—The murine FDCPmix cell line was maintained and induced to differentiate essentially as described previously (23). Briefly, cells were maintained in Fischers medium with 10% donor horse serum and interleukin-3 and subcultured every 3–4 days. Neutrophilic differentiation was induced by addition of interleukin-3, granulocyte macrophage-colony stimulating factor, and granulocyte-colony stimulating factor and the cells allowed to develop for 7 days. Monocytic/macrophage differentiation was induced by culturing cells in interleukin-3, granulocyte-macrophage-colony stimulating factor, and macrophage-colony stimulating factor for 10 days. Following completion of the differentiation program cells were harvested and total RNA prepared using Trizol. The success of the differentiation was confirmed by morphological analysis of the cells following cytospinning and staining with May Grunwald and Giemsa (data not shown).

**Generation of cDNA from Lineage Marker-depleted Hemopoietic Cells**—For generation of lineage-depleted cells, murine bone marrow cells were depleted of lineage marker-bearing cells using cell surface markers specific for B cells (B220), T cells (CD4 and CD5), macrophages/monocytes (Mac1), and granulocytes (Gr1). The residual subpopulation of cells that were negative for the above lineage markers were therefore enriched for primitive hemopoietic cells. This population

is referred to in the text as the lin<sup>-</sup> population. mRNA was generated from bone marrow and the lin<sup>-</sup> population using the Invitrogen micro-fast track mRNA kit and cDNA produced using the Invitrogen copy kit. Each of the cDNA preparations were sheared with *AluI* and linkers were ligated on to allow PCR amplification of the cDNA populations. It is important to note that different linkers were used for each cDNA preparation to minimize the chances of carry-over or cross-contamination between the different cDNAs during PCR. The cDNAs were amplified by PCR and used in Southern blotting experiments to assess gene expression. Success of the sorting and cDNA generation exercise has been confirmed previously (24).

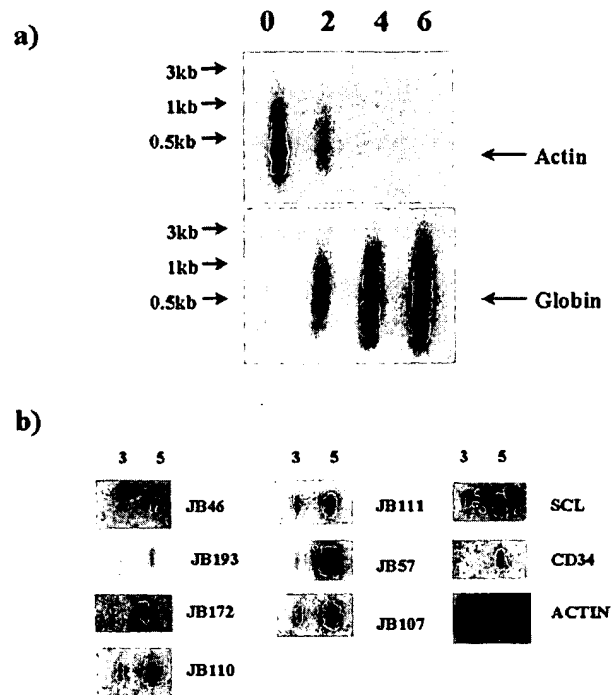
**Generation of RNA from Lineage Restricted Hemopoietic Cells**—Separately, individual lineages were enriched using specific anti-surface marker antibodies. The antibodies used were TER-119 for erythrocytes (PharMingen), CD41 for megakaryocytes (PharMingen), Gr-1 for granulocytes (Cambridge Bioscience), CD11b for monocytes and macrophages (PharMingen), and CD3 for T-lymphocytes (Cambridge Biosciences). Cells were isolated using Dynabeads according to the manufacturers instructions, the cells washed, lysed, and RNA prepared using Trizol. JB542 expression was analyzed by PCR using the following primers which are expected to yield a product of 386 base pairs: 5'-CACATTCATATCCCCGTGAGG-3' and 5'-CCTCATCAACTTGGT-GCTG-3'. PCR was carried out for 30 cycles as described previously (24).

**EB *In Situ* Hybridization**—Procedures for *in situ* hybridization of EB sections were derived from hybridization protocols for mouse embryo sections (25, 26) and from whole mount *in situ* hybridization procedures on Ebs.<sup>2</sup> Briefly, EBs allowed to differentiate *in vitro* for various periods of time were fixed in 4% paraformaldehyde, embedded into paraffin wax, and cut into 7- $\mu$ m sections. Hybridization conditions were as described (25). Single-stranded riboprobes for  $\alpha$ -globin were synthesized as run-off transcripts from linearized plasmid templates under standard conditions, essentially as described (27). Signal was visualized with alkaline phosphatase-conjugated anti-DIG antibodies and NBT/BCIP color substrate.

## RESULTS

**Generation of a Day 3/Day 5 Subtracted Library**—To investigate the usefulness of differentiating ES cells as a source of known and novel hemopoietic gene products we have performed subtractive hybridization using cDNA populations obtained from day 3 and day 5 embryoid bodies. We have used intact embryoid bodies as our cDNA source in an attempt to ensure inclusion of both hemopoietic and stromal/supportive cell gene products in the subtracted library. Prior to the initiation of the subtractive hybridization, the successful generation of HSCs within the EBs was confirmed by measurement of "transiently engrafting" CFU-A stem cell activity in the day 5 EBs as described previously (19). Subtractive hybridization was carried out using the "Gene Expression Screen" method of Wang and Brown (21) as described under "Experimental Procedures." The success of the subtractive hybridization is shown in Fig. 1a which demonstrates effective removal of common  $\beta$ -actin sequences after 4 rounds of subtraction and substantial up-regulation of hemopoietic specific  $\alpha$ -globin gene sequences over the six rounds of subtraction. Slot-blot analysis (Fig. 1b) of a number of cDNAs from the subtracted library has demonstrated only low numbers (less than 10%) of non-day 3/day 5 differentially expressed cDNAs, thus further confirming the success and completeness of the subtraction. Also shown in Fig. 1b is differential expression of the primitive hemopoietic markers *Scl* (28, 29) and *CD34* (30) confirming the usefulness of the day 3/day 5 time frame as a source of known genes of importance to hemopoietic stem cell generation and function.

**Differentiating ES Cells Are a Rich Source of Known and Novel Hemopoietic Gene Products**—We have sequenced 474 cDNAs from this subtracted library and data base searching has demonstrated 132 of these sequences to be identical to cDNAs already deposited in the non-EST data bases. Of these 132 cDNAs, which are representative of 80 discrete gene prod-



**FIG. 1. Confirmation of the success of the subtractive hybridization.** a, Southern blots of cDNA from alternate stages of the subtractive hybridization showing actin suppression and  $\alpha$ -globin enrichment over the six rounds of subtraction. Agarose gels were run of 5  $\mu$ g of cDNA from every second round of the subtractive hybridization. The gels were blotted and probed with actin or  $\alpha$ -globin, both of which were labeled by random priming. b, slot-blot analysis of day 3/5 differential expression of unknown and known cDNAs in the subtracted library. Slot-blot analysis was prepared with 200 ng of day 3 or day 5 EB-derived cDNA. These blots were probed with clone specific probes labeled by random priming.

ucts, 61 (representative of 31 discrete gene products) are known to be primarily expressed in, and to function in, hemopoietic or hemopoietic supportive cells (Table I). Among the hemopoietic cell-associated sequences is a number characteristic of primitive hemopoietic cells and these include the transcription factors *Ikaros* (31) and *GFI-1b* (32), the surface markers *CD34* (30), *PECAM* (33), and the integrins  $\alpha_4$  and  $\beta_3$  (34) and the primitive cell associated GDP dissociation inhibitor (35). In addition, the transcription factor *Tel* identified in the library has a known role in HSC migration during development and may functionally relate to the CXCR4 chemokine receptor in this context (36, 37). The presence of these cDNAs in the subtracted library, along with the demonstration of differential expression of *CD34* and *Scl* (Fig. 1b) further confirms the usefulness of the ES cell differentiation system as a source of known genes of importance to primitive hemopoietic cell function. The hemopoietic supportive cell-associated cDNAs include a number of sequences associated with bone marrow stromal cells such as the integrin ligands collagen, fibronectin, and laminin (34), endothelial cell-associated sequences such as VE-cadherin (38) and angiopoietin (39), and the adipocytic cDNAs *Paf-1* and apolipoprotein-B. The remaining 71 (46 discrete gene products) identifiable sequences show no clear commonality in their tissue affiliations and appear to have little in common in either their known functions or expression patterns (Table II). Thus this preliminary sequencing data suggests that ~46% of identifiable cDNAs in the subtractive library are of relevance to the process of hemopoiesis with 38% being of hemopoietic cell origin and 8% being derived from presumed hemopoietic supportive cells. The fact that there is no other major tissue-

<sup>2</sup> U. Menzel, personal observations.



TABLE I  
List of known haemopoietic and haemopoietic related gene products in the subtracted library (for this and subsequent tables, numbers in brackets represent numbers of repeats of the individual sequences)

Clone No.	Accession No.	Description
<b>Haemopoietic</b>		
JB536	NM_001773	Murine <i>CD34</i>
JB287	L03547	Murine <i>Ikaros</i> transcription factor
JB311	AF017275	Murine <i>Gfi-1B</i> transcription factor (2)
JB340	L07918	Murine <i>GDP</i> dissociation inhibitor (5)
JB82	U19617	Murine <i>Elf-1</i> transcription factor
JB524	Y07915	Murine <i>TEL</i> transcription factor
JB218	P06802	Plasma cell membrane glycoprotein ( <i>PC-1</i> ) (4)
JB192	P03979	TCR $\gamma$ -chain V region PT-gamma- $\frac{1}{2}$ precursor
JB140	X97399	Murine Procathepsin E
JB245	L35933	Murine erythrocyte membrane protein 4.2 (3)
JB110	X78709	Murine <i>NRFI</i> transcription factor
JB71	P04919	Band 3 anion exchange protein ( <i>MEB3</i> )
JB50	X64594	50-kDa Erythrocyte plasma membrane glycoprotein
JB57	NM_008816	Murine <i>PECAM</i>
JB48	M15268	Murine 5-aminolevulinic acid synthetase (2)
JB6	S76831	Murine tropomodulin (4)
JB442	S32537	Murine erythroid transcription factor <i>NF-E2</i> (3)
JB40	M26897	Murine $\epsilon$ -globin (4)
JB29	M26894	Murine $\beta$ -H1-globin (8)
JB21	S71555	Murine $\beta$ globin (2)
JB532	AF078905	Murine $\zeta$ globin
JB195	U34627	Murine $\alpha$ -4 integrin
JB534	AF026509	Murine integrin $\beta$ -3 subunit
<b>Haemopoietic related</b>		
JB18	M14423	Murine pro- $\alpha$ -1 type 1 collagen (3)
JB31	M85212	Murine tartrate resistant acid phosphatase
JB65	Q09163	Preadipocyte factor-1
JB70	M14081	Apolipoprotein B
JB107	X83930	Murine VE-cadherin
JB226	U43541	Murine laminin $\beta$ -2 (2)
JB342	U82612	Fibronectin
JB535	AF125176	Murine angiopoietin related

specific gene expression represented in the library indicates that hemopoiesis is the predominant developmental process occurring in the EBs between days 3 and 5 of differentiation.

In addition to the known genes outlined in Tables I and II there are 78 ESTs for which no expression or functional data is yet available (Table III) and 264 sequences that currently have no counterparts in any publicly available data base. Of these 342 ESTs or unidentifiable sequences, it is likely, given the percentages of known genes that are of relevance to hemopoiesis that ~150 of these novel cDNAs/ESTs will represent genes of importance to the development and maintenance of hemopoiesis. As many as 125 are likely to be expressed specifically in hemopoietic cells.

Clearly, it is not feasible to pursue the full-length cDNA cloning and biological characterization of all of these novel cDNAs and thus our strategy has been to concentrate on the characterization of cDNAs that are novel but that have motifs that allow an assessment of likely family affiliation or function. Using this, and a range of expression analysis strategies, we have identified a number of novel transcription factors and signaling molecules that are preferentially expressed in primitive hemopoietic cells. As a demonstration of the usefulness of this strategy in identifying novel cDNAs expressed in HSCs and potentially of relevance to HSC generation and function, we have pursued the cloning and the characterization of the expression profile of one of the novel cDNAs (JB542). This cDNA was selected for study as the fragment present in the library had homology with members of an interferon inducible family of transmembrane proteins and was thus identified as a potential novel surface marker of HSCs. A cDNA incorporating the full-length coding sequence for JB542 was obtained from a day 5 EB cDNA library as described under "Experimental Procedures." This sequence is shown in Fig. 2a and reveals a

450-base pair cDNA encoding a 134-amino acid peptide with a calculated molecular mass of 14,667 daltons. Interestingly, while this cDNA incorporates the full-length coding sequence for JB542, the size of the primary transcript for JB542 in tissues blots (see Fig. 3) is ~2.1 kilobases suggesting the presence of extensive 5'- and 3'-untranslated sequences. As mentioned, data base searching revealed similarities with members of an interferon inducible gene family typified by the 9-27/*leu* 13 (40) and 1-8D (41) proteins (Fig. 2b) the roles for which remain to be elucidated. The highest levels of similarity is with a membrane protein of undetermined function from the marbled electric ray, *Torpedo marmorata* (42). EST data base searching with this full-length cDNA also reveals a close human homologue encoded by an embryo-derived EST (accession number AA463818) which displays ~81% identity with the murine protein (Fig. 2b). This human JB542 sequence is incorporated within a genomic sequence from chromosome 11q15 (accession number AF015416). The predicted murine protein is highly charged with acidic and basic residues accounting for 20% of the total sequence. The overall charge is +3. To investigate the likely orientation of the JB542 protein within the membrane we have performed Kyte-Doolittle analysis which reveals (data not shown) a potential transmembrane region, extending approximately from amino acids 40 to 60. There is a further potential transmembrane region predicted between amino acids 90 and 110 and thus determination of the precise orientation of JB542 within the cell membrane awaits epitope tagging studies which are underway in our laboratory.

To attempt to implicate JB542 in hemopoietic cell function we have examined its expression in bone marrow, spleen, and a range of other tissues. Results from such tissue blot analysis demonstrates that expression of JB542 is predominantly seen in the brain and bone marrow with lower level expression in

TABLE II  
List of known non-haemopoietic gene products in the subtracted library

Clone No.	Accession No.	Description
JB1	U75215	Mouse neutral amino acid transporter
JB2	AJ006278	Acetylglucosaminyltransferase-like protein (2)
JB8	U24674	Mouse <i>Mxi1</i>
JB22	D67076	Mouse <i>ADAMTS-1</i>
JB24	X03351	Prealbumin (2)
JB27	U09816	G <sub>M2</sub> activator protein
JB53	Y12582	Mouse calpain-like protease
JB69	X95825	Mono-ADP ribosyltransferase
JB76	D49914	Mouse seryl t-RNA synthase
JB112	L25338	Mouse folate-binding protein
JB113	X52634	tlm oncogene
JB123	L05781	Cytosolic epoxide hydrolase
JB147	X07201	<i>H19</i> gene (2)
JB153	X84301	MLE transposase (2)
JB157	P54729	BS4 protein
JB181	X98330	Cardiac ryanodin receptor
JB330	U18869	Mouse mitogen responsive phosphoprotein p96
JB188	J04970	Carboxypeptidase M (2)
JB201	U49968	Lipoma preferred partner
JB209	AB004109	Phosphatidylserine synthase
JB211	U29726	MAP kinase
JB215	M80783	Human B12 protein (TNF $\alpha$ induced)
JB227	U80040	Human nuclear Aconitase
JB240	L09159	Selenium-dependent/RHOA proto-oncogene (5)
JB256	AB004231	Selenium-dependent glutathione peroxidase (5)
JB274	A5587	Human TNF type-1 receptor associated protein (2)
JB279	U53586	Mouse <i>Evi-5</i>
JB283	D89678	Human mRNA for A-U rich element-binding protein
JB289	AF004211	Mouse <i>Et</i> homeodomain protein
JB326	AF042379	Human spindle pole body protein, spc97 homolog
JB331	U48830	Mouse subtilisin-like proprotein convertase-7
JB349	X84896	Mouse ATP receptor (P2X gene) (12)
JB394	U49393	Mouse sarcoplasmic reticulum Calcium ATPase, <i>SERCA 3b</i>
JB399	D10727	Mouse <i>NDPP-1</i> protein
JB422	X57971	Mouse gap junction gene, connexin 37
JB436	U24493	Mouse tryptophan 2,3-dioxygenase
JB476	Q13618	Human Cullin homology 3 ( <i>Cul-3</i> )
JB485	U20238	Mouse GTPase activating protein ( <i>GAP111</i> )
JB486	X99807	Mouse selenoprotein P
JB495	L25602	Mouse bone morphogenetic protein 2 ( <i>BMP-2</i> )
JB253	U26348	Mouse insulin-like growth factor receptor
JB531	M55154	Mouse transglutaminase
JB528	AF231120	Murine iron-regulated transporter <i>IREG1</i>
JB529	M27073	Murine protein phosphatase type 1
JB530	AF078776	Human p53-binding protein
JB391	NM008093	Murine <i>GATA-5</i> transcription factor

testes and skeletal muscle (Fig. 3a). To further examine the expression of JB542 within the hemopoietic system and attempt to investigate any primitive cell-restricted expression patterns, we have adopted two cellular models. First, we have examined expression in the primitive murine hemopoietic cell line, FDCPmix, which displays many phenotypic similarities to murine transiently engrafting stem cells (23, 43). These cells can self-renew under the proliferative stimulus of interleukin 3 and can be induced to differentiate along a range of hemopoietic lineages following treatment with appropriate growth factors. For the purposes of the present study we have produced RNA from parental FDCPmix cells and from these cells following differentiation along the monocytic and granulocytic pathways. Analysis of expression of JB542 reveals it to be expressed in the parental FDCPmix cells suggesting expression in primitive hemopoietic cells (Fig. 3b). Furthermore, this expression appears to be seen predominantly in undifferentiated cells as upon differentiation along either the monocytic or granulocytic pathways, expression drops markedly (Fig. 3b). To further confirm this preferential expression of JB542 in primitive hemopoietic cells, we have examined expression in normal murine bone marrow cells and in populations of cells enriched for primitive cells by depletion of cells bearing lineage markers (lin<sup>-</sup>). As can be seen in Fig. 3c, JB542 is expressed at much

higher levels in the lin<sup>-</sup> cells than in the total bone marrow cells and while the lin<sup>-</sup> population does represent a purified population of stem cells, it is enriched for primitive stem and progenitor cells and thus expression in this population is further suggestive of preferential JB542 expression in primitive hemopoietic cells. To examine the hemopoietic expression further, we have separated hemopoietic cells into their component lineages using immunomagnetic techniques and have assessed JB542 expression in these sorted cell populations. As shown in Fig. 4, PCR again reveals preferential expression in lineage negative cells compared with lineage positive cells. The expression in lineage positive cells appears to be accountable for by maintained expression in erythrocytes, megakaryocytes, and granulocytes. In contrast expression is lost following commitment to and differentiation down the macrophage or T cell lineages. These data therefore confirms the primitive cell expression of JB542 but suggests that its down-regulation is lineage dependent.

Thus analysis of the expression patterns of JB542, a novel cDNA identified within the ES cell subtracted library has demonstrated its preferential brain and hemopoietic expression patterns and furthermore, has revealed that the hemopoietic expression is preferentially seen in primitive cell types. This identification of this novel hemopoietic cDNA confirms the

TABLE III

List of EST sequences in the subtracted library (sequences marked by asterisks represent murine homologues of the indicated human ESTs)

Clone No.	Accession No.	Clone	Accession No.	Clone	Accession No.
JB10	AA200396	JB111	AA620185	JB365	AA671643
JB13	AA063843	JB128	AB002368	JB408	AA656848(2)
JB14	AA103447	JB129	AA409088(2)	JB433	AF03726*
JB19	AA273417	JB136	AA079440*	JB440	AA08753
JB20	AA557474*	JB137	AA436394	JB452	AA124089
JB23	AA187926	JB138	AA118441	JB462	AA26596
JB30	AA260787	JB149	AA402272*	JB470	AA959838
JB32	AA111447	JB174	Z35720*	JB477	AA656464
JB33	AA211956	JB182	AA516655	JB501	D89077
JB34	AA166323	JB183	AA636355	JB516	AA008378
JB37	AA553221	JB193	C81256	JB517	AV382276
JB41	AA075665*	JB205	AA412981(3)	JB518	AW495347
JB42	H35148	JB207	AA388214	JB519	AI548540
JB58	AA591032	JB210	T32446*	JB520	AA387113
JB59	AA197359	JB213	AA451978*	JB521	AF276682
JB63	AA185198	JB235	AA555722	JB125	D80000
JB64	AA407284	JB247	AA171007	JB128	AB002368
JB66	W59213	JB254	AA245632	JB236	D87448
JB72	AA120208	JB290	AA109999	JB244	D87448
JB74	HI7599*	JB299	W13517	JB309	Q14999
JB75	AA253285*	JB308	AA681812	JB357	Q87743
JB84	AA125052	JB312	AA771001	JB460	AB002334
JB94	AA240945	JB360	AA537736	JB522	AB032974
JB100	AA065822	JB363	AA155047(2)	JB526	D86970
JB527	AV375824				

usefulness of the *in vitro* ES cell system as a valuable source of novel genes of relevance to primitive hemopoietic cell function.

**Analysis of the ES Cell Subtracted Library Reveals Preferential Expression of Erythroid Lineage Gene Products**—As mentioned above, there is a number of primitive cell-restricted cDNA sequences identified in the subtracted library confirming its value as a source of cDNAs of relevance to immature hemopoietic cell generation and function. In keeping with the previously reported absence of mature cells or lineage committed progenitors from the EBs at day 5 (19), there are no cDNA markers of mature myeloid cells and only two markers of the lymphoid lineages (T cell receptor  $\gamma$  and plasma cell membrane glycoprotein). Curiously, however, a large cohort of the identifiable cDNAs is representative of genes that are not characteristic of primitive hemopoietic cells but are more typically associated with maturing and mature erythrocytes. These erythroid genes include the transcription factors *NRF1* (44) and *NFE2* (45), the enzymes *5-ALAS* (46) and procathepsin E (47), the surface markers (see Ref. 48 for a review of erythrocyte surface markers) erythrocyte membrane protein 4.2 (49), 50-kDa plasma membrane glycoprotein (50), and Band-3 anion exchange protein (51), the tropomodulin structural gene (52) as well as multiple globin sequences. We have previously demonstrated the absence of erythroid progenitor cells and markers of mature erythropoiesis in the day 5 EBs and have used benzidine staining to demonstrate that mature, benzidine-positive erythrocytes do not emerge in the EB differentiation system until day 8. The absence of evidence of maturing and mature erythroid cells in the EBs at day 5 has led us to tentatively conclude that while a multigenic program is evident in the differentiating ES cells, the relative wealth of erythroid genes is suggestive of erythroid lineage preconditioning of the ES cell-derived hemopoietic stem cell. It remains possible that this erythroid lineage gene expression is indicative of emergence of small numbers of committed erythroid cells in the EBs at day 5 that may be hard to detect using conventional bioassays. However, Northern blot (data not shown) and *in situ* (Fig. 5) analyses of  $\alpha$ -globin expression in the developed EBs has revealed expression at time points even earlier than day 5. Indeed Fig. 5 shows that  $\alpha$ -globin species are readily detectable in day 4 EBs and weakly in day 3 EBs. In our hands these time

points are prior to the emergence of transiently engrafting stem cells or any identifiable committed progenitors. Indeed day 3, at which time low levels of globin expression is detectable, precedes the emergence of long-term repopulating stem cells in the ES cell system (19) and is more in keeping with the time of emergence of hemangioblasts (17).

In summary these data are consistent with the initiation of erythroid gene expression coincident with the emergence of the earliest detectable hemopoietic stem cells in the ES cell *in vitro* differentiation system. It is our contention that these data supports a model of erythroid preconditioning of the hemopoietic stem cell in the developing embryoid bodies.

**Adult, Definitive HSCs Are Not Exclusively Preconditioned to Erythropoiesis**—It is possible that this apparent erythroid preconditioning is a feature of primitive embryonic type HSCs and may therefore not be shared by adult definitive HSCs which have previously been reported to display a multigenic program of gene expression (53). To examine this issue we have arrayed many of the erythroid and primitive cell genes identified in the ES cell subtracted library (columns 1 and 2 and 3 and 4 of Fig. 6a, respectively) as well as genes that are more typically representative of differentiating and differentiated myeloid cells (columns 5 and 6 of Fig. 6a). We have used these arrays to examine gene expression in FDCPmix cells and lin<sup>−</sup> primary hemopoietic cells with a view to assessing the similarities in the multigenic gene expression pattern between these adult cells sources and the ES cell-derived HSC. RNA was prepared from FDCPmix cells, converted to cDNA, labeled, and used to probe the dot blots outlined on Fig. 6a. The results shown in Fig. 6b reveal that in the parental FDCPmix cell line, in contrast to the ES cell-derived hemopoietic cells, a multigenic program of gene expression is evident with as expected, a good representation of stem cell-associated gene expression alongside expression of a number of erythroid and myeloid genes but with no clear predisposition to erythroid gene expression. A similar multigenic expression pattern without evidence of erythroid pre-conditioning is seen in primary murine lineage-negative cell populations (Fig. 6c) further indicating that, in contrast to the ES cell-derived HSCs, adult HSCs either from primary sources or as a self-renewing cell line, do not display significant erythroid preconditioning but instead exhibit a mul-

**A**

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1  ctcagctagg aagacacggc gctggaaccc atggacacct catatccccg
      M D T S Y P R
51  tgaggacccc cgggctccat catccccgaa ggctgatgct gcagcccaca
      E D P R A P S S R K A D A A A H
101 cagccctctc catgggaaca cctggcccta caccacgaga tcacatgctc
      T A L S M G T P G R T P R D H M L
151 tgggtctgtc tcagcacgat gtacctgaat ctgtgctgcc ttggattcct
      W S V F S T M Y L N L C C L G F L
201 ggcgctggtc cactctgtca aggcccgaga ccagaagatg gctgggaact
      A L V H S V K A R D Q K M A G N
251 tggaggctgc aaggcagtat ggctccaaag ccaagtgcta caacatcctg
      L E A A R Q Y G S K A K C Y N I L
301 gctgcaatgt ggacattggt gccccattg ctgctcctgg gactgggtgg
      A A M W T L V P P L L L L G L V V
351 gactggcgcc ttgcacctgt ccaagttagc caaagactct gcggctttct
      T G A L H L S K L A K D S A A F
401 tcagcaccaa gtttgatgag gaggactata actaagagtt ccgagcctgt
      F S T K F D E E D Y N
451

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**B**

	10	20	30	40	50	60	70
Murine JB542:	MDTSYPREDP	RAPSSRKADA	AAHTALSMGT	PGRTPRDHML	WSVFSTMYLN	LCCLGFLALV	HSVKARDQKM
Human JB542:	MDTAYPREDT	RAPTPSKAGA	--HTALTG	PHPPPRDHLI	WSVFSTLYLN	LCCLGFLALA	YSIKARDQKV
Torpedo 14k:	ME-Y--RTDQ	-VPMSPRSVQ	GAP-----	GTLPIRDHLP	WSIFNLFYMN	VCCLGLTAMI	FSVKSRRDKV
Human 9-27:	MHKEEHEVAV	LGAPPSTILP	RSTVINIH--	SETSVDPHV	WSLFNTLFLN	WCCLGFIAFA	YSVKSRRDKM
Human 1-8D:	MLKEEQEVAM	LGGPHNPAPP	TSTVIHIR	SETSVDPHV	WSLFNTLFMN	TCCLGFIAFA	YSVKSRRDKM
JB542 con.	MDT-YPREDE	RAP---KA-A	--HTAL----	P---PRDH--	WSVFST-YLN	LCCLGFLAL-	-S-KARDQKM
Family con.	M			DH	WS F	N CCLG A	S K RD K

	80	90	100	110	120	130	
Murine JB542:	AGNLEAARQY	GSKAKCYNIL	AAMWTLVPPL	LLLGLVVTGA	LHLSKLAKDS	AAFFSTKFDE	EDYN
Human JB542:	VGDLAARRF	GSKAKCYNIL	AAMWTLVPPL	LLLGLYYTGA	LHLARLAKDS	AAFFSTKFDD	ADYD
Torpedo 14k:	VGDVEGARHY	GSTARSLNIA	ATVLGILLII	ILIGLAATGT	IQALKYG		
Human 9-27:	VGDVTGAQAY	ASTAKCLNIW	ALILGILMTI	GFILSLVFGS	VTVYHIMLQI	IQEKRKY	
Human 1-8D:	VGDVTGAQAY	ASTAKCLNIW	ALILGIFMTI	LLVIIPVLVV	QAQR		
JB542 con.	-G-LEAAR--	GSKAKCYNIL	AAMWTLVPPL	LLLGL--TGA	LHL--LAKDS	AAFSTKFD-	-DY-
Family con.	G A	S AK NI A					

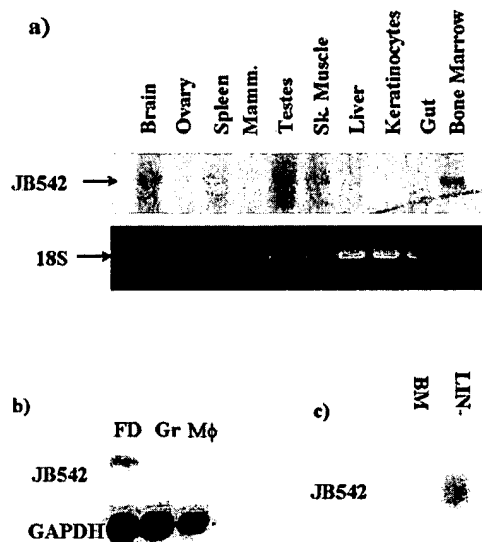
Fig. 2. cDNA sequence and homology comparisons for JB542. A, the full-length cDNA for JB542 is shown. This has been derived from a day 5 EB cDNA library using primers internal to the cloned fragment and primers from vector sequences flanking the cDNA insert (accession number AJ009781). Note that the putative transmembrane regions are underlined. B, homology between murine and human JB542 and other members of the interferon inducible family of membrane proteins. The human and murine JB542 consensus sequence is shown (JB542 con) as well as the overall family consensus (Family con).

tigenic expression program. These data is therefore in agreement with that of Hu *et al.* (53) who have used PCR to demonstrate a similar multigenic program in FDCPmix and sorted primary stem cells (53). The FDCPmix gene expression pattern changes on differentiation along the neutrophilic lineage with a loss of erythroid and stem cell gene expression and a concomitant increase in expression of some myeloid genes, most notably lysozyme (data not shown). These data suggests that the apparent erythroid specific preconditioning of the ES cell-derived stem cells may be an indication of their primitive rather than definitive nature and that definitive stem cells, either as a homogenous cell line or as an enriched population of lineage-negative cells display a more multigenic gene expression pattern. Further evidence pointing to the primitive nature of the EB-derived hemopoiesis is the predominance of fetal, or primitive,  $\alpha$ - and  $\beta$ -globin species (Ref. 54 and Table I). These results therefore suggest that erythroid gene expression detected at the earliest time of emergence of primitive hemopoietic cells in the *in vitro* ES cell system is indicative of erythroid

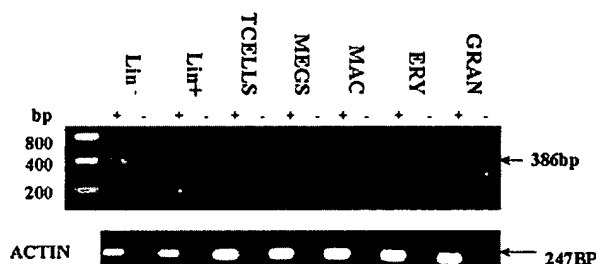
preconditioning of the primitive hemopoietic stem cell and may go some way to explaining the rapidity of mature primitive erythrocyte generation from primitive HSC and the apparent preference for erythroid differentiation in nongrowth factor-treated ES cells (9, 55).

#### DISCUSSION

The practical limitations inherent in studies using adult bone marrow stem cells have precluded directed analysis of the gene products involved in regulating stem cell production and function. For this reason we have turned our attention to the ES cell system as an *in vitro* model of developmental hemopoiesis. Other studies have also capitalized on the usefulness of this *in vitro* differentiation system to identify novel protein kinase cDNAs (56, 57) and to characterize a number of hemopoietic and developmental genes (58). We now report that precise definition of the time frame of emergence of primitive hemopoietic stem cells in the EBs (19) has allowed us to use this *in vitro* differentiation system to generate subtracted

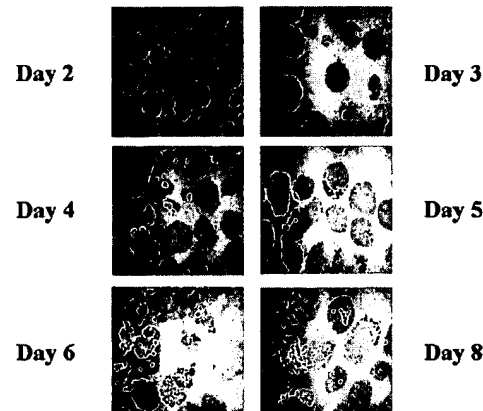


**FIG. 3. JB542 is expressed in hemopoietic tissues with preferential expression in immature cells.** *a*, expression of JB542 in murine tissues: RNA from the tissues shown was prepared and 20  $\mu$ g of each run on a denaturing agarose gel. This gel was then blotted and the blot probed with JB542-specific probes. As indicated normalization was by comparison with the ethidium bromide stained 18 S ribosomal RNA band. *b*, expression of JB542 in primitive hemopoietic cells: FDCPmix cells were grown and allowed to differentiate along either the granulocytic or monocytic pathways. Total RNA was prepared and Northern blotted in an attempt to detect JB542 in parental or differentiated FDCPmix cells. *c*, also JB542 expression was examined in sorted primitive (lineage negative) murine hemopoietic cells. cDNA was prepared from lineage negative cells and from bone marrow and this was run out on an agarose gel and Southern blotted. Both the Northern and Southern blots in this figure were probed with random primed JB542 sequences.



**FIG. 4. Examination of JB542 hemopoietic expression.** Murine bone marrow cells were sorted into individual lineages and into lin- and lin+ cell populations using immunomagnetic separation techniques. RNA was prepared from these cell populations and expression of JB542 assessed by PCR. PCR was allowed to run for 30 cycles following which the products were visualized on a 1.2% agarose gel. Actin has been included as a loading control.

cDNA libraries that are abundant sources of both known and novel hemopoietic and hemopoietic related gene products. Our analysis of 474 sequences from the subtracted library suggests that ~46% of identifiable sequences are likely to be of relevance to the processes of hemopoiesis. The hemopoietically derived known genes include a number of markers of primitive cells such as *CD34* (30) and additionally we have shown differential expression of *SCL* (28, 29), a transcription factor known to function at a most fundamental level in hemopoietic development (59, 60). Intriguingly, despite the absence of detectable mature hemopoietic cells in the EBs, a large number of the hemopoietic gene products differentially expressed in concert with the emergence of stem cells in the EBs are more typically associated with mature elements of the erythroid lineage. We have interpreted this as indicating that, within the multigenic



**FIG. 5.  $\alpha$ -Globin is detected at the earliest time points of hemopoietic commitment in developing EBs.** EBs at days 2, 3, 4, 5, 6, and 8 were sectioned and probed for  $\alpha$ -globin expression using an *in vitro* transcribed digoxigenin-labeled probe.

program of the differentiating EBs, there is evidence of an erythroid preconditioning of the primitive cells generated in the EBs. It remains possible that small numbers of committed erythroid progenitors are present in the EBs at day 5, however, if this was the case these would be the only lineage committed cells present in the EBs and would again argue for some pre-disposition to erythroid development. We have used *in situ* hybridization and Northern blotting to demonstrate that globin gene sequences are detectable at day 4 and even as early as day 3 although only weakly. These time points precede the emergence of transiently engrafting stem cells in the *in vitro* differentiation system arguing for expression of globin in relatively uncommitted cells perhaps even hemangioblasts (17). This erythroid preconditioning appears to be a hallmark of primitive hemopoietic cells as both analysis of gene expression patterns in immature adult hemopoietic cells and also assessment of the precise nature of the globin species identified within the subtracted cDNA population attests to the primitive nature of the erythropoiesis being detected. It is intriguing that during development, primitive erythrocytes appear very rapidly following appearance of the first primitive HSC within the yolk sac (55) and in differentiating ES cells is the preferred hemopoietic lineage for terminal differentiation. This preconditioning seen in the EB-derived primitive HSC may help to account for this phenomenon, *i.e.* that the stem cells are generated with a specific predisposition for the erythroid lineage. Why should this pre-conditioning be lost in the adult HSCs? It is possible that the reason for the apparent pre-conditioning in the EBs and the earliest embryonic HSC is that they see no other growth factors at these early developmental stages and thus are free to spontaneously commit to their preordained lineage. In the later embryo and in the adult, the emergence of growth factor expression may subvert this preconditioning and allow a more multigenic program to be activated within the HSC. This is clearly what we have seen and mirrors the data from others indicating a wide gene expression pattern in primitive adult hemopoietic cells (53, 61, 62).

The data clearly indicate that hemopoiesis is by far the predominant developmental process occurring over the day 3/5 time frame analyzed and no other major tissue-specific gene expression patterns are detected among the known genes outlined in Table II. We have previously reported that there are few detectable HSC per EB at day 5 (19), however, the gene expression level detected in this study and particularly the diffuse nature of the globin staining patterns observed following *in situ* analysis suggest that hemopoietic commitment is considerably more widespread than we had first believed. It is

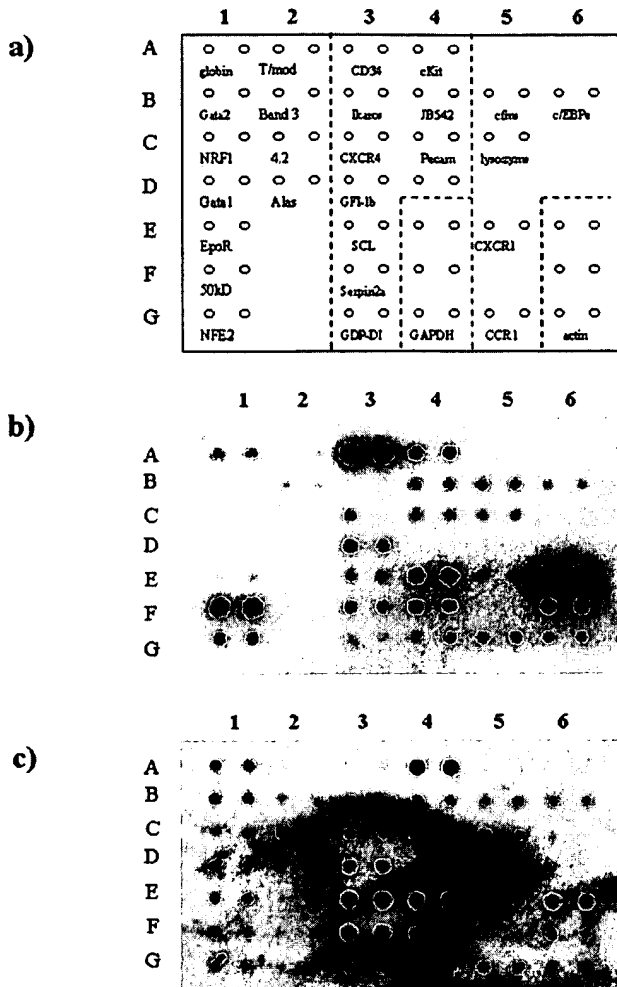


FIG. 6. Dot-blot analysis of erythroid, stem cell, and myeloid cell cDNA expression reveals a multigenic pattern of gene expression in FDCPmix and lineage negative primary cells. *a*, template showing the layout of the dot blots. Each dot has 100 ng of cDNA per spot. cDNAs are abbreviated as outlined in the text. Actin and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) are included as loading controls with actin being loaded at 50, 5, and 0.5 ng/spot and glyceraldehyde-3-phosphate dehydrogenase at 100, 10, and 1 ng/spot. Thus the differential intensity of the actin and glyceraldehyde-3-phosphate dehydrogenase spots correlates with these different loadings. *b*, dot-blot probed with radiolabeled total cDNA from FDCPmix cells. *c*, dot-blot probed with radiolabeled total cDNA from sorted lineage negative primary murine hemopoietic cells.

likely that while limited numbers of HSC are detected by *in vitro* assay, hemopoietic gene expression is more widespread within the EB and a number of cells are expressing hemopoietic genes. It may be that only a few of the cells express the appropriate cohort of genes to allow them to be fully functional stem cells although further more rigorous analysis of EB HSC numbers is required before definitive conclusions can be reached.

We have also demonstrated the ES cell system to be a valuable source of novel hemopoietic genes and describe the cloning and characterization of JB542, a novel hemopoietic cell surface protein with expression patterns indicative of primitive hemopoietic cell expression. Embryologically this transcript is detectable by PCR between days 9.5 and 11.5 but the levels are too low to allow precise *in situ* localization (data not shown). A JB542 human homologue with 81% identity to the murine protein is also described and resides on human chromosome 11. We are currently raising antibodies to JB542 with a view to examining the specific clonogenic nature of the cells expressing

this cell surface protein.

In conclusion, therefore, we have demonstrated the murine ES cell system to be a very valuable source of both known and novel hemopoietic and hemopoietic related genes. Furthermore analysis of known genes is suggestive of erythroid preconditioning of primitive hemopoietic stem cells.

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**3073609CD1\_PRT\_5\_PF-0673-USN**

132 aa

**IFITM1**

125 aa

Interferon-induced transmembrane protein 1, a putative receptor signaling protein that plays a role in the transduction of antiproliferative and homotypic adhesion signals, regulates L-selectin (SELL) expression

Match: Length=72, Identity: 51%, Similarity:75%, Query Overlap: 54%, Subject Overlap: 50%, E-value:1.5e-21, Score:82

Query: 35 DHLIWSVFSTLYLNLCCLGFLALAYSIKARDQKVVGDLAARRFGSKAKCYNILAAMWTL 94  
DH++WS+F+TL+LN CCLGF+A AYS+K+RD+K+VGD+ A+ + S AKC NI A  
Sbjct: 35 DHVVWSLFNTLFLNWCCLGFI AFAYSVKSRDRKMVGDTVGAQAYASTAKCLNIWA----- 89

Query: 95 VPPLLLLGLVVT 106  
L+LG+++T  
Sbjct: 90 ----LILGILMT 97

Schematic Colors:

Very Strong	Strong	High	Moderate	Low	Weak
>95%	80-95%	45-80%	35-45%	25-35%	20-25%



# Powers and Pitfalls in Sequence Analysis: The 70% Hurdle

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**H**igh-throughput technologies impress us almost every week with novel global results and big numbers. They often reveal important general trends that are impossible to realize with classical, low-throughput experimental methods, yet (so far) they provide fewer insights into specific, molecular detail. Because of the amount of data involved, high-throughput technologies imply the use of bioinformatics methods that deal with information transformation, storage, and analysis. By necessity, most of these processes are automated.

Partly because of the nature of current publication schemes, the accuracy and error margins of a given method are often only found in small print. It is obvious that each method has its limits and also that during data processing, some information will be lost or diluted. Because of the current need to integrate and add value to data, results from high-throughput experiments (if made publicly accessible) are often taken further by third-party research that relies on the quality of these data. Thus, I believe that public awareness of error margins for high-throughput experimental and computational methods should be increased; the incredibly valuable data accumulating in various heterogeneous databases permit powerful analyses but should not be overinterpreted. In the following discussion, I will concentrate on limits in computational sequence analysis, which is far from being perfect (Table 1), despite the fact that sequencing itself is highly automated and accurate, and despite the fact that sequence information is described in simple linear terms (using a four-letter alphabet). On

average, a 70% accuracy just to predict functional and structural features has to be considered a success (Table 1).

## Limitations in the Total Knowledge Base of Protein Function

As these analysis methods are knowledge based, one of the reasons for the inaccuracy is that the quality of data in public sequence databases is still insufficient (e.g., Bork and Bairoch 1996; Bhatia et al. 1997; Pennisi 1999). This is particularly true for data on protein function. Protein function is loosely defined; cellular function is more than the very complicated network of individual molecular interactions on which it is based (Bork et al. 1998). Furthermore, the semantics for functional features are not always established. For instance, the notion of a "protein complex" not only depends heavily on detection and purification methods—which, in turn, are constantly evolving—but also on environmental conditions. Protein function is context dependent, and both molecular and cellular aspects have to be considered (for review, see Bork et al. 1998).

To illustrate some of this complexity, a good example is lactate dehydrogenase: This gene product can act both as a dehydrogenase and an eye lens structural protein, depending on its context (for review, see Piatigorsky and Wistow 1991). Even without the complication of a second, unrelated role for the same gene product, do we know enough about the function of lactate dehydrogenase, one of the best-studied proteins? We know its biochemical pathway (at least in human and some model organisms), its different isoenzymes (in organisms) with different context-dependent

properties, its regulation, and the organization of its quaternary structure. However, we are probably still missing much information, even on crucial molecular features: Are we sure about alternative splice variants? Can we exclude age-dependent post-translational modifications in some tissues? Our knowledge is even more limited regarding higher order functions that involve concentration, compartmental organization, dynamics, regulation, and perhaps even the impact of external environment. Often, the available data give at best some reliable qualitative results on functional features but far from a complete understanding of functionality. Yet our ability to annotate genome sequences and translate information therein relies heavily on the summaries of features attached to each sequence in the respective public databases.

## Limitations of Gene Expression Data Extrapolations

As more high-throughput technologies follow, the data will become more complicated than sequences. Novel complementary data types such as gene expression arrays will generate more functional information, but conclusions from these data are often stretched with regard to protein products. The expression of genes and their reciprocal proteins seems to correlate weakly, with a correlation coefficient of 0.48 (Anderson and Seilhammer 1997). Furthermore, recent studies (Hanke et al. 1999; Mironov et al. 1999) show that alternative splicing might affect >30% of the human genes, although measurements at the protein level have yet to confirm this. Finally, the number of known post-

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**Table 1. Selected Examples of Prediction Accuracy in Different Areas of Sequence Analysis**

Prediction of	Acc $\times$ cov <sup>a</sup>	Accuracy (%)	Coverage or coverage in % of reference set	Reference <sup>b</sup>
Human promoters	0.35	50	70% of annotated test set	Prestidge 1995; P. Bucher (pers. comm)
Human regulatory RNA elements	0.34	85	40% of new DNA	Dandekar and Sharma (1998)
Human genes (only presence)	0.49	70	70% of chromosome 22	Dunham et al. (1999) and refs. therein
Human SNPs by EST comparison	0.21	70	30% of all proteins with SNP	Buelow et al. (1999); Sunyaev et al. (2000)
Human alternative splicing	0.45	90	50% of all splice sites	Hanke et al. (1999)
Transmembranes (only presence)	0.85	85	99% of annotated test set	Tusnady and Simon (1998) and refs. therein
Signal peptides (only presence)	.90	90	100% of annotated test set	Nielsen et al. (1999)
GPI ancors (incl cleavage site)	.72	72	100% of annotated test set	Eisenhaber et al. (1999)
Coiled coil (only presence)	.81	90	90% of annotated coiled coil	Lupas (1996)
Secondary structure (Three states)	.77	77	100% of 3D test set	Jones (1999) and refs. therein
Buried or exposed residues	.74	74	100% of 3D test set	Rost (1996)
Residue hydration	.72	72	100% of 3D test set	Ehrlich et al. (1998)
Protein folds (in Mycoplasma)	.49	98	50% of Mycoplasma ORFs	Teichmann et al. (1999) and refs. therein
Homology (several methods)	.49	98	50% of 3D test set	Muller et al. (1999) and refs. therein
Functional features by homology	.63	90	70% unicellular genomes	Bork and Koonin (1998); Brenner (1999)
Function association by context	.25	50	10% high confidence in yeast	Marcotte et al. (1999b)
Cellular localization (two states)	.77	77	100% of annotated test set	Andrade et al. (1998)

The numbers referred to are in many cases crude estimates taken or sometimes even estimated from the literature and have an expected accuracy of ~70%. Direct comparison of the numbers might be misleading as the context is not properly explained here. Furthermore, although most of the examples are two state predictions, the percentage numbers do not take into account random occurrences of the states. All test sets are most likely biased (e.g., current 31) test sets do not contain many compositionally biased regions, which probably contain up to 15% of all residues, and annotation test sets are far from being perfect; see text), i.e., the real accuracy is thus probably lower.

<sup>a</sup>To make the numbers more comparable, accuracy has been multiplied by coverage; some methods give accuracy for different degree of coverage and roughly justify this procedure. However, often it is biased toward sensitivity as specificity cannot be properly taken into account. Most features predicted with an accuracy  $\times$  coverage >0.70 are of structural nature and at best only indirectly imply a certain functionality.

<sup>b</sup>Only one recent reference is given and if indicated, references therein should also be considered as other reports do not always agree with the numbers given.

translational modifications of gene products is increasing constantly, so that the complexity at the protein level is enormous. Each of these modifications may change the function of the respective gene products drastically. (The entire aspect of context-dependent gene regulation is excluded from current discussions as we are only beginning to understand the complex underlying genetic machinery. For example, promoter prediction in eukaryotes has a success of only ~35% (Table 1), and there are many other regulatory elements that we cannot predict at all.)

#### Limitations Created by Third-Party Analyses

Public releases of completely sequenced genomes exceed a rate of one per month, with thousands of function predictions therein. Gene annotation via sequence database searches is already a routine job, but even here the error rate is considerable (Table 1). The lower limit of errors in current functional annotation of large-scale sequencing projects is 8% (Brenner 1999). As errors accumulate and propagate (Bork and Bairoch 1996; Bhatia et al 1997; Smith and Zhang

1997; Bork and Koonin 1998; Pennisi 1999), it becomes more difficult to infer correct function from the many possibilities revealed by a database search. Increasing these complications is the fact that computer programs often cannot even retrieve the source of the stored information (Doerks et al. 1998).

#### Use of Complementary Information to Limit Errors in Function Prediction

Some new information can be retrieved from completely sequenced genomes, for example, function can be predicted by exploitation of genomic context.

Based on the observation that interacting proteins in one organism sometimes have homologs in other organisms fused together in a single gene, Marcotte et al. (1999a) predicted novel interactions for 50% of yeast proteins using gene fusion information. However, they noted an overlap with classical methods and an error rate of 82%. To see a signal they had to correct for domains present in many proteins (Marcotte et al. 1999a). By considering only orthologs with fission and fusion events (Enright et al. 1999, Snel et al. 2000), the signal-to-noise ratio increases and the number of predictions drops dramatically (7% of *Escherichia coli* proteins; Enright et al. 1999). With a particular question in mind, Does protein X have interaction partners?, the generation of hypotheses is extremely useful; yet to provide a general overview of protein function, it is advisable to keep the errors small. Further information can be added later, which is easier than retracting stored information. But how do we incorporate the information on error margins? Such estimates (sometimes not even the sources of the annotation) are not visible in current databases that store the results of computational approaches.

### Taking the 70% Hurdle

As noted above, most prediction schemes extrapolate from current knowledge, and many bioinformatics methods have difficulty exceeding a 70% prediction accuracy (numbers in Table 1 are often overestimates because the test sets used are usually not representative of all sequences). On one hand, current methods seem to capture important features and explain general trends; on the other hand, 30% of the features are missing or predicted wrongly. This has to be kept in mind

when processing the results further. Also the 70% accuracy often attaches to methods that deal with discrete objects such as sequences; making estimates about the prediction of cellular features is much more difficult as one first has to agree on semantics (or ontology in a database sense) to describe complex processes in a comparable way.

All of the above focuses on limitations in the computational prediction of qualitative features. There remains a long way to go until we are able to describe molecular processes quantitatively; current simulations of complex systems are still very rough and simplistic. However, there is still no doubt that sequence analysis is extremely powerful and that the generation of hypotheses derived by computational methods will be more and more often the first successful step in the design of experiments. If 70% of such experiments were successful, the speed of scientific discoveries would grow exponentially.

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# Tyrosine Kinase-Dependent Regulation of L-Selectin Expression Through the Leu-13 Signal Transduction Molecule

## Evidence for a Protein Kinase C-Independent Mechanism of L-Selectin Shedding<sup>1</sup>

Margaret Frey, Michelle M. Appenheimer, and Sharon S. Evans<sup>2</sup>

The L-selectin adhesion molecule mediates lymphocyte extravasation in peripheral lymph nodes, and has also been implicated in directing leukocyte recruitment to inflammatory tissues and metastasis of lymphoid malignancies. In this study, we demonstrate a novel level of regulation of L-selectin expression that involves the 16-kDa Leu-13 signal transduction molecule. Leu-13 is a member of a multimeric cell surface complex in lymphocytes that includes TAPA-1 (target of antiproliferative Ab-1, CD81) as well as lineage-specific proteins. In the present study, mAb-induced ligation of Leu-13 was shown to rapidly down-regulate L-selectin surface density on normal and malignant human lymphocytes, and to markedly inhibit L-selectin-mediated adhesion of lymphocytes to soluble carbohydrate ligands (i.e., PPME, phosphomonoester core polysaccharide) and to lymph node high endothelial venules. Through the use of genistein and staurosporine, potent inhibitors of tyrosine kinases (TK) and protein kinase C (PKC), respectively, Leu-13-induced L-selectin down-modulation was demonstrated to involve a TK-dependent, PKC-independent pathway, and was attributed to increased L-selectin shedding from surface membranes. Notably, direct L-selectin ligation, modeling cross-linking interactions with endothelial cell ligands, similarly down-regulates L-selectin surface expression through a TK-dependent, PKC-independent mechanism. In sharp contrast, PMA and anti-CD3 mAb down-regulate L-selectin via a staurosporine-sensitive, genistein-resistant pathway that is closely linked to lymphocyte proliferation. Taken together, these results demonstrate a novel role for Leu-13- and L-selectin-induced TK activity in control of L-selectin expression, thus providing insight into the complex molecular mechanisms that potentially regulate L-selectin-dependent lymphocyte homing in vivo. *The Journal of Immunology*, 1997, 158: 5424-5434.

Immune surveillance is dependent on the efficient, continuous trafficking of lymphocytes between the blood and peripheral tissues. The L-selectin (CD62L) leukocyte adhesion molecule mediates the initial attachment and slow rolling of lymphocytes along specialized postcapillary high endothelial venules (HEV)<sup>3</sup> within peripheral lymph nodes and Peyer's patches (1, 2). This initial adhesion event occurs under high shear forces within blood vessels and is a critical first step for entry of lymphocytes into lymphoid tissues. L-selectin on lymphocytes, neutrophils, and monocytes has also been implicated in the development of pathologic processes such as metastasis of lymphoid tumors (3-5) and the recruitment of immune effector cells to sites of acute and chronic

inflammatory responses in autoimmune diseases, as well as during graft rejection and ischemia-reperfusion injury (1, 2, 6, 7).

L-selectin expression and, thereby, lymphocyte homing potential, are tightly regulated at multiple levels during B and T cell maturation and development. During lymphocyte maturation in the bone marrow, L-selectin synthesis and expression by B and T lymphocytes are programmed to coordinate with the exit of these cells into the circulation, such that expression is highest on the most mature CD19<sup>+</sup>CD20<sup>+</sup> bone marrow B cells and on CD7<sup>+</sup>CD2<sup>+</sup>CD34<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> prothymocytes (8, 9). Recent studies have indicated that the restricted localization of newly synthesized L-selectin on lymphocyte microvilli is another important determinant in regulating lymphocyte interactions with endothelial cells (1, 2, 10). The physiologic factors that regulate L-selectin synthesis in vivo are not known. The immunomodulatory cytokines IFN- $\alpha$  and IFN- $\beta$  have been demonstrated to increase L-selectin gene expression, cell surface levels, and ligand-binding activity in vitro (11),<sup>4</sup> suggesting that these cytokines or related molecules control L-selectin synthesis in vivo. L-selectin is also susceptible to rapid down-regulation in vivo and in vitro. Recent studies suggest that during initial lymphocyte-endothelial cell interactions, low level shedding of L-selectin contributes to the velocity of leukocyte rolling along the luminal surface of blood vessels (12). Related to these observations, L-selectin cross-linking by mAb, potentially mimicking L-selectin-ligand interactions, has been shown to down-modulate L-selectin surface levels on lymphocytes

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<sup>3</sup> Abbreviations used in this paper: HEV, high endothelial venules; PE, phycoerythrin; PKC, protein kinase C; PPME, phosphomonoester core polysaccharide; QR, quantum red; sL-selectin, shed L-selectin; TAPA, target of antiproliferative antibody; TK, tyrosine kinase.

<sup>4</sup> M. M. Appenheimer, P. Silva, R. A. Bruce, S. O. Gollnick, P. Aplan, and S. S. Evans. Identification of the 5'-transcriptional regulatory region of the interferon- $\alpha$  inducible human L-selectin gene.

in vitro (13, 14). Within tissues, B lymphocyte activation and proliferation in lymph node germinal centers are associated with decreased L-selectin expression (15, 16). Similarly, B or T lymphocyte activation in vitro by PMA (5, 14, 17–19) B cell growth factors and Ig cross-linking (16), IL-2 (20), and anti-CD3 mAb (21) induces L-selectin shedding from the cell surface. Although the signaling events regulating L-selectin shedding are largely undefined, studies using protein kinase inhibitors have implicated a PKC-dependent pathway in PMA-mediated down-modulation of this adhesion molecule from the lymphocyte surface (5, 14, 18, 21).

The lymphocyte cell surface molecules Leu-13 and TAPA-1 (CD81) are expressed in a complex with lineage-specific molecules (i.e., B cell-restricted molecules, CD19, CD21; T cell-restricted molecules, CD4, CD8) (22–26), and have been suggested to play an important role in controlling cell-to-cell interactions within lymphoid tissues. The 16-kDa Leu-13 cell surface molecule expressed by B and T lymphocytes (22–30) and by vascular endothelium (31, 32) is encoded by the IFN- $\alpha$  or  $\beta$ - and IFN- $\gamma$ -inducible 9-27 gene (25, 28, 29, 32, 33). Non-covalent interactions between Leu-13 and the 26-kDa TAPA-1 molecule, a member of the tetraspan family of cell surface molecules, as well as other complex members have been demonstrated by immunoprecipitation analysis (22–27). Ligation of these individual molecules with specific mAb induces cellular aggregation through undefined adhesion pathways (22, 25, 27–30, 34) that may serve to amplify the immune response by promoting adhesion of responding cells to APC (i.e., dendritic cells, monocytes, B cells) or by enhancing T-B cell interactions. Ab binding to Leu-13 as well as to other members of this multimeric complex has also been shown to inhibit growth factor-driven proliferation of normal and malignant lymphocytes (22, 25, 27, 29), further supporting the notion that these molecules function as integral components of an important signal transduction complex in immune effector cells. This signaling complex appears to be closely linked to tyrosine phosphorylation events in lymphocytes, based on evidence that homotypic adhesion initiated by mAb specific for Leu-13, TAPA-1, CD19, and CD21 is inhibited by tyrosine kinase (TK) inhibitors (28, 30) and by mAb specific for the CD45 tyrosine phosphatase (34), a known regulator of TK activity in lymphocytes. In addition, ligation of members of this complex (TAPA-1, CD19) reportedly initiates rapid tyrosine phosphorylation of a number of cellular proteins (35, 36).

The present study was undertaken to examine the relationship between the L-selectin adhesion molecule and the Leu-13 signaling complex. Our studies demonstrate a novel level of regulation of L-selectin expression that involves the Leu-13 signal transduction molecule. Leu-13 ligation by mAb was shown to initiate TK-dependent, PKC-independent down-regulation of L-selectin surface density on B and T lymphocytes, and to inhibit L-selectin-mediated adhesion of lymphocytes to vascular endothelial cell ligands. Furthermore, direct L-selectin cross-linking by the DREG56 mAb was similarly shown to initiate L-selectin shedding through a TK-dependent, PKC-independent signal transduction pathway, closely paralleling Leu-13-mediated effects. In sharp contrast, PMA- and anti-CD3 mAb-induced L-selectin down-regulation was prevented by staurosporine, a potent PKC inhibitor, but not by the TK inhibitor genistein. Taken together, these data indicate that L-selectin down-regulation and shedding from the lymphocyte cell surface can be triggered through multiple independent intracellular signaling pathways that differentially depend on PKC or TK activity, thus providing insight into the complexity

of the mechanisms that potentially control L-selectin expression, and thereby lymphocyte homing potential in vivo.

## Materials and Methods

### Cell isolation and cultures

PBMC were isolated from normal donor buffy coat leukocyte concentrates (American Red Cross, Buffalo, NY Region office) by Ficoll-Hypaque centrifugation, as described previously (28, 29). Following removal of adherent cells, the PBL population was cultured at a final concentration of  $4 \times 10^6$  cells/ml in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% FCS (Life Technologies), 2 mM L-glutamine, 100 U/ml penicillin, and 50  $\mu$ g/ml streptomycin.

An IFN-sensitive subclone of the Burkitt's lymphoma-derived Daudi B cell line was kindly provided by Dr. A. Hovanessian (Pasteur Institute, Paris, France). Cells were maintained in logarithmic growth ( $5-9 \times 10^5$  cells/ml) in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 50  $\mu$ g/ml streptomycin, and  $10^{-5}$  M  $\beta$ -mercaptoethanol (Sigma Chemical Co., St. Louis, MO) (11, 28). Since Daudi cells constitutively express low levels of L-selectin and Leu-13, it was necessary to incubate these cells with rIFN- $\alpha$  (500 IU/ml for 24 h) to up-regulate Leu-13 and L-selectin levels, as described (11, 28). Human IFN- $\alpha$  ( $2.2 \times 10^6$  IU/ml) was kindly provided by Dr. P. Trotta (Schering Corp., Bloomfield, NJ).

### Monoclonal antibodies

The Leu-13-specific mAb, a murine IgG1, was previously described by Dr. R. Evans (Millard Fillmore Hospital, Buffalo, NY) (27). The 13A5 mAb, a murine IgM specific for the Leu-13 protein (25), was a kind gift of Dr. G. Deblandre (University of Brussels, Rhode-St-Genese, Belgium). Anti-CD3 mAb (OKT3) were kindly provided by Dr. R. Evans. Anti-B<sub>4</sub>, an IgG1 mAb specific for the CD19 Ag, was kindly provided by Dr. L. Nadler (Dana-Farber Cancer Institute, Boston, MA). Control murine mAb A4 (IgG1), which does not react with human cells, was generously provided by Dr. R. Ward (Roswell Park Cancer Institute, Buffalo, NY). The L-selectin-specific mAb DREG56 (17) was kindly provided by Dr. E. Butcher (Stanford University, Stanford, CA). The following Ab were obtained commercially from Becton Dickinson (Sunnyvale, CA): L-selectin-specific mAb Leu-8 FITC and Leu-8 PE, anti-CD20 FITC, anti-CD5 PE, anti-CD19 PE, anti-Leu-2a (CD8) FITC, anti-Leu-5b (CD2) FITC, anti-Leu-3a (CD4) FITC, anti-Leu-4 (CD3) FITC, and FITC- or PE-conjugated isotype-matched control mAb. FITC-conjugated anti-L-selectin (DREG56), anti-CD44, and anti-CD45 were obtained from Immunotech (Westbrook, ME); anti-L-selectin mAb TQ1 and isotype-matched control mAb (IgG1) were obtained from Coulter Corp. (Hialeah, FL); anti-human LFA-1 (CD11a) FITC was obtained from Endogen (Boston, MA); anti-human CD19 quantum red (QR) and mouse IgG1-QR were obtained from Sigma Chemical Co.; and goat F(ab')<sub>2</sub> anti-mouse IgG FITC was obtained from Cappel Products (Durham, NC). Selected mAb which bind to distinct L-selectin epitopes (i.e., anti-Leu-8, TQ1, DREG56) were used as indicated, based on optimal activity in specific assays.

### Reagents and kinase inhibitors

PMA and PHA were purchased from Sigma Chemical Co. Staurosporine, a potent inhibitor of PKC activity, was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). Genistein, a specific TK inhibitor, was purchased from Life Technologies. PKC-specific inhibitors, calphostin C and chelerythrine chloride, were purchased from Kamiya Biomedical Co. and Calbiochem (San Diego, CA), respectively. H-7, a PKC- and protein kinase A-specific inhibitor, was purchased from Seikagaku Kogyo Co. (Tokyo, Japan).

### Flow-cytometric analysis

Direct immunofluorescence analysis of the relative expression of lymphocyte cell surface molecules was determined using a FACScan (Becton Dickinson), as described previously (11, 28, 29). A total of  $10^6$  cells was washed with PBS/0.02% sodium azide and then incubated with 0.1 mg/ml of mouse Ig (Sigma Chemical Co.) for 10 min at 4°C to block FcR sites. Cells were then incubated with saturating amounts of fluorochrome-labeled mAb or isotype-matched control mAb for 30 min at 4°C, washed in PBS/sodium azide, and fixed in 1% formaldehyde/PBS. Indirect immunofluorescence analysis of Leu-13 expression was performed as described (28, 29). A total of 10,000 events was collected, and analysis was performed using Winlist 1.0 (Verity Software House, Topsham, ME).

L-selectin expression on normal human peripheral blood B cell (CD19<sup>+</sup>CD3<sup>-</sup>) and T cell (CD3<sup>+</sup>CD19<sup>-</sup>) populations was determined by

multiparameter flow-cytometric analysis of PBL stained simultaneously with anti-CD3 FITC, anti-Leu-8 PE, and anti-CD19 QR. PBL stained individually with fluorochrome-labeled mAb were used to compensate for overlap of fluorescence emission spectra. Parallel samples were stained simultaneously with isotype-matched fluorochrome-labeled (FITC, PE, and QR) mAb as a negative control.

#### PPME-binding assay

Analysis of L-selectin-dependent binding of lymphocytes to PPME, the phosphomonoester core from *Hansenula hostii* phosphomannan, was performed as described (11, 37). Following culture under the indicated conditions, PBL were washed once in RPMI 1640 medium, resuspended at  $5 \times 10^6$  cells/ml, and then incubated for 15 min at 4°C either in medium alone or with 10 mM EDTA, the L-selectin-specific mAb TQ1 (10 µg/ml), or an isotype-matched control Ab (10 µg/ml). Without washing, cells were then incubated with a 1/200 dilution of fluorescein-conjugated PPME (generous gift of Dr. L. Stoolman, University of Michigan, Ann Arbor, MI) for 30 min at 4°C, and then analyzed immediately by flow cytometry. Fluorescence measurements of FITC-conjugated PPME binding were made with logarithmic amplification.

#### Stamper-Woodruff frozen section assay

Lymphocyte binding to HEV was assessed essentially as described (17, 38). Following culture under the indicated conditions, human PBL were resuspended at  $5 \times 10^7$  cells/ml in complete medium, and then incubated with or without the L-selectin-specific blocking mAb DREG56 (50 µg/ml) for 30 min at room temperature. Human PBL ( $5 \times 10^6$  PBLs in 100 µl) were then overlaid onto 12-µm-thick cryosections of BALB/c lymph nodes mounted on glass slides. Previous studies have established that L-selectin-binding specificity is maintained during assay of human lymphocyte adhesion to mouse lymph node HEV (17). Slides were rotated at 112 rpm (Labline Instrument, Melrose Park, IL) at 4°C for 30 min, and nonadherent cells were removed by gentle washing in cold PBS. Slides were fixed vertically in 3% glutaraldehyde/PBS for 1 h, permeabilized in 70% ethanol, and stained with 0.5% toluidine/absolute ethanol. A total of 300 to 500 HEV was examined by light microscopy, and data are expressed as the mean number of lymphocytes bound per HEV  $\pm$  SD; each sample was assayed in triplicate. Exogenous human lymphocytes exhibit a darkly stained, round appearance, and thus were distinguished easily from the histologically distinct murine tissue lymphocytes and HEV.

#### Lymphocyte activation assay

[<sup>3</sup>H]TdR incorporation was used to measure lymphocyte activation and proliferation, as described (25, 29). Cells were plated in 96-well plates ( $2 \times 10^5$  cells/well) and then pulsed 4 h with 0.6 µM of [<sup>3</sup>H]TdR (6.7 Ci/mmol; DuPont NEN, Boston, MA). Samples were harvested using a PhD cell harvester (Cambridge Technology, Watertown, MA) and counted in a Beckman scintillation counter (Beckman Instruments, Wakefield, MA). The proliferation index was calculated based on the relative [<sup>3</sup>H]TdR incorporation detected in cultures incubated with medium alone.

#### Analysis of shed L-selectin by ELISA

Shed L-selectin (sL-selectin) levels in culture supernatants were determined in replicate wells using a sandwich ELISA (Bender Med Systems, Vienna, Austria). The DREG56 mAb used in cross-linking experiments partially interfered (<25% inhibition) with the detection of L-selectin in the ELISA. Therefore, L-selectin standard curves were generated in the presence of 5 µg/ml of DREG56 mAb for analysis of sL-selectin levels in culture supernatants from cells incubated with the identical concentration of L-selectin cross-linking mAb. The anti-Leu-8 mAb, which also induces L-selectin down-regulation from the cell surface, was completely inhibitory in the ELISA, and thus, the effects of this mAb on L-selectin shedding could not be evaluated.

## Results

#### Leu-13 ligation by mAb down-regulates L-selectin surface expression on peripheral blood B and T lymphocytes and on Daudi B lymphoma cells

The effects of anti-Leu-13 mAb, a murine IgG1, on L-selectin cell surface expression on human peripheral blood CD19<sup>+</sup>CD3<sup>+</sup> B cells and CD3<sup>+</sup>CD19<sup>+</sup> T cells, as well as on the Burkitt's lymphoma-derived Daudi B cell line, were determined by multiparameter flow-cytometric analysis. Since Daudi B cells constitutively express low

levels of both L-selectin and Leu-13, it was necessary to preincubate these cells with IFN- $\alpha$  for 24 h to up-regulate these molecules, as described previously (11, 28), before the addition of anti-Leu-13 mAb. For comparison, L-selectin down-regulation from lymphocyte surface membranes was also examined in response to PMA, a known activator of L-selectin shedding (12, 18, 19, 39, 40).

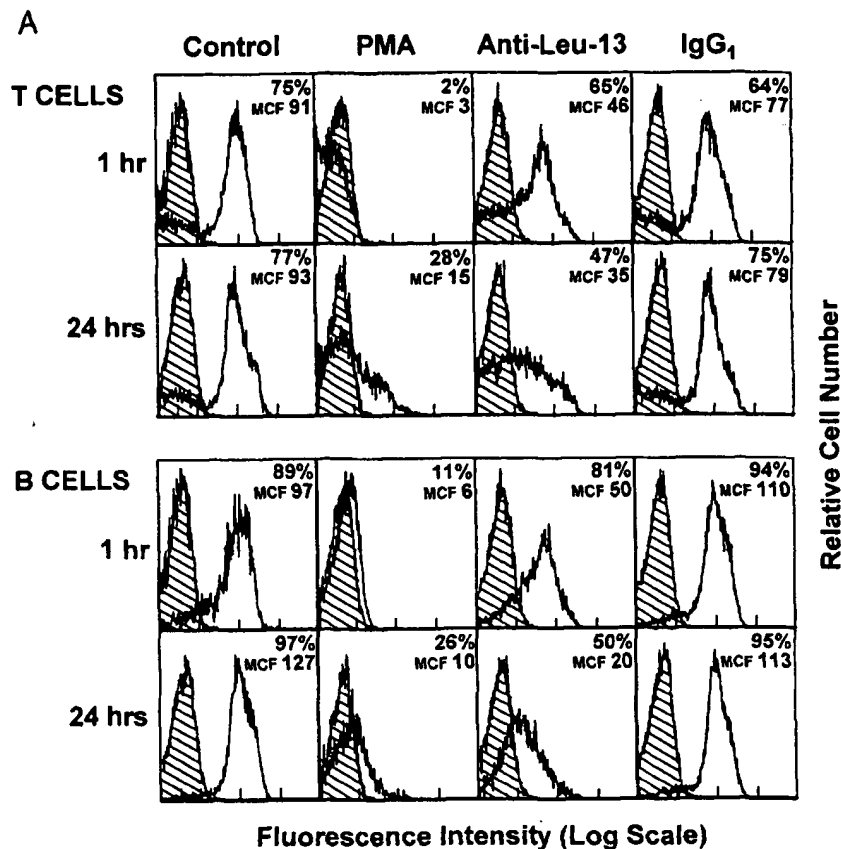
Consistent with previous reports, lymphocyte stimulation by PMA for 1 h triggered nearly complete loss of cell surface L-selectin on peripheral blood B and T lymphocytes and on Daudi B cells; slight recovery of L-selectin expression was observed following exposure to PMA for 24 h (Fig. 1). Anti-Leu-13 mAb also triggered significant down-regulation of L-selectin surface density as well as the number of L-selectin-positive cells in peripheral blood B and T lymphocyte populations (Fig. 1A) and in Daudi cells (Fig. 1B), although the kinetics were delayed compared with the PMA response. In this regard, partial reduction of L-selectin expression was consistently observed following incubation of human lymphocytes with anti-Leu-13 mAb for 1 h. Further reduction of L-selectin expression by normal and malignant lymphocyte populations occurred during continuous exposure to anti-Leu-13 mAb over a 24-h period, such that only a small proportion of cells expressed L-selectin at a low density. In contrast, an isotype-matched control mAb, A4 (an IgG1), had no effect on L-selectin surface expression (Fig. 1A). The Leu-13-induced inhibitory effects on L-selectin expression were observed at 37°C, but not at 4°C (not shown), indicating that these results do not reflect steric hindrance of L-selectin epitopes by anti-Leu-13 mAb. Moreover, L-selectin down-regulation was not enhanced by subsequent cross-linking of bound anti-Leu-13 mAb by secondary Ab (not shown). Anti-B<sub>4</sub>, a murine IgG1 mAb that recognizes CD19 surface molecules, but does not trigger cellular homotypic aggregation responses (30), did not inhibit L-selectin expression in Daudi cells (not shown), indicating that the observed anti-Leu-13 mAb effects are not mediated solely by FcR pathways.

#### Effect of anti-Leu-13 mAb on the expression of B and T lymphocyte surface molecules

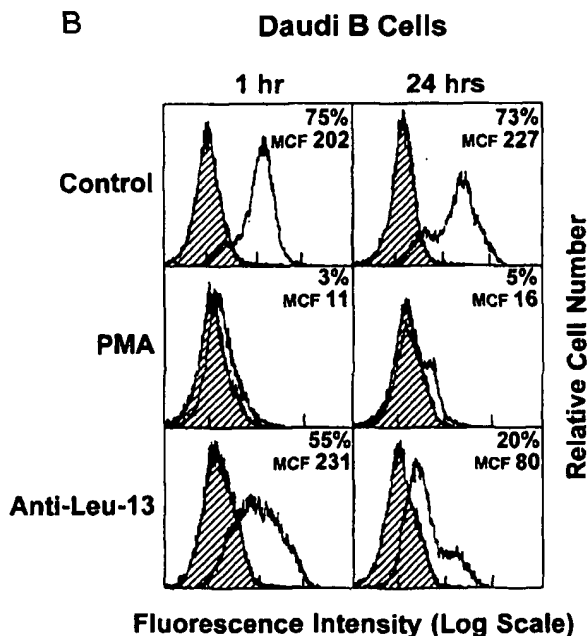
Studies were performed to determine whether the inhibitory effect of anti-Leu-13 mAb was restricted to the regulation of L-selectin, or whether this signaling molecule also influenced the expression of other cell surface molecules. To address this question, human PBL were incubated in the absence or presence of anti-Leu-13 mAb for 24 h, and then stained with mAb specific for a wide panel of cell surface molecules and analyzed by flow cytometry. In the experiment shown in Figure 2, surface molecule expression was analyzed on total PBL populations, with the exception of CD19 and CD20. Since B cells represent minor populations within total PBL (i.e., 5–10%), it was necessary to gate on CD19<sup>+</sup> cells or CD20<sup>+</sup> cells to analyze the effects of anti-Leu-13 mAb on the expression of these B cell surface proteins.

Anti-Leu-13 mAb was again found to significantly reduce the density of L-selectin on human lymphocytes (Fig. 2). Anti-Leu-13 mAb also down-regulated expression of the Leu-13 protein, such that 24 h after the addition of mAb, only very low levels of Leu-13 could be detected. These data further support the notion that the inhibition of L-selectin density observed following incubation with anti-Leu-13 mAb is not due to steric hindrance by the respective mAb, since at 24 h, when maximal L-selectin down-regulation is detected, Leu-13 and its associated mAb are essentially absent from the cell surface.

Anti-Leu-13 mAb had essentially no effect on lymphocyte expression of the adhesion molecules CD44 and CD11a (LFA-1), and had only minimal effects on CD2 (LFA-2) expression.



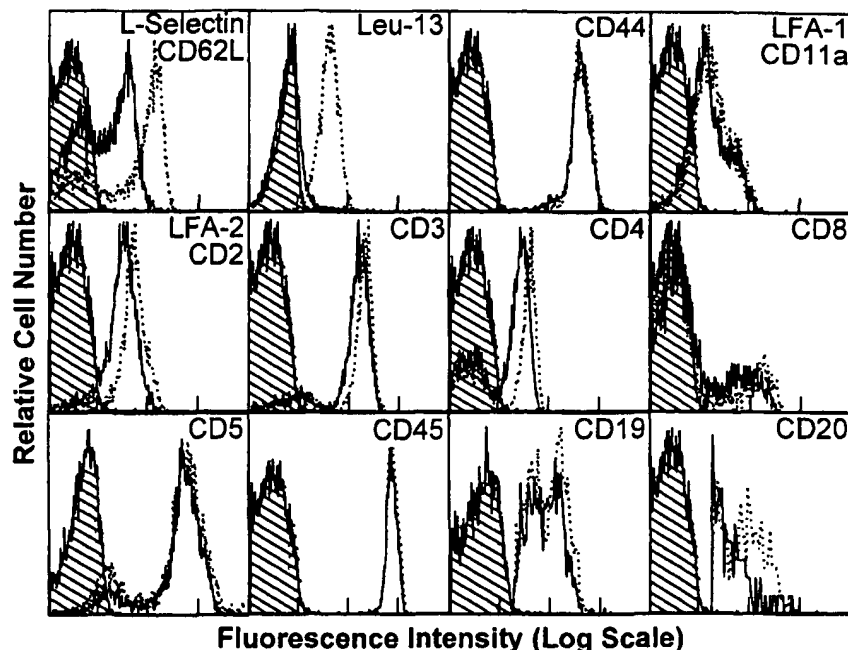
**FIGURE 1.** Leu-13 cross-linking by mAb down-regulates L-selectin surface expression by human lymphocytes. *A*, Peripheral blood B and T lymphocytes were incubated in medium alone (control) or with 50 ng/ml PMA, 10  $\mu$ g/ml anti-Leu-13 mAb, or 10  $\mu$ g/ml of an isotype-matched control mAb, A4, for 1 or 24 h, and then stained with anti-Leu-8 PE. Identical results were obtained using DREG56-FITC-labeled L-selectin-specific mAb (not shown). L-selectin expression on CD3<sup>+</sup>CD19<sup>-</sup> T cells and CD19<sup>+</sup>CD3<sup>-</sup> B cells was analyzed by multiparameter flow cytometry. *B*, IFN- $\alpha$ -treated Daudi B cells were incubated for 1 or 24 h in medium alone (control) or with 50 ng/ml PMA or 10  $\mu$ g/ml anti-Leu-13 mAb, stained with anti-Leu-8 FITC, and analyzed by flow cytometry. Hatched histograms represent the background fluorescence of cells stained with FITC-labeled isotype-matched negative control mAb. The frequency and mean channel fluorescence (MCF) of L-selectin-positive cells are indicated. Note the marked decrease in L-selectin surface density on T and B lymphocytes following incubation with anti-Leu-13 mAb. Consistent results were observed in >10 independent experiments.



Moreover, anti-Leu-13 mAb had only limited effects on the expression of the T cell surface molecules CD3, CD4, CD8, or CD5. Anti-Leu-13 mAb also did not affect CD45 density, a cell surface tyrosine phosphatase that has been implicated recently in the Leu-13 signal transduction pathway (34). In peripheral blood B cells, anti-Leu-13 mAb appeared to induce a partial downward

shift in the fluorescence intensity of anti-CD20 staining profiles, but did not markedly affect CD19 levels. Taken together, these data suggest that the down-modulatory effects of anti-Leu-13 mAb on L-selectin expression do not reflect general inhibitory effects on all lymphocyte cell surface molecules, but rather are restricted to a limited number of lymphocyte plasma membrane proteins.

**FIGURE 2.** Flow-cytometric analysis of the effects of anti-Leu-13 mAb on lymphocyte expression of cell surface proteins. Human PBL were cultured in medium alone (dotted histogram) or with 10  $\mu$ g/ml anti-Leu-13 mAb (solid histogram) for 24 h, stained with fluorochrome-labeled mAb specific for the indicated cell surface molecules (with the exception of Leu-13), and analyzed by flow cytometry. Leu-13 surface expression was determined by indirect immunofluorescence staining. Hatched histograms represent the background fluorescence of cells stained with fluorochrome-labeled isotype-matched control mAb. Data are representative of three independent experiments.

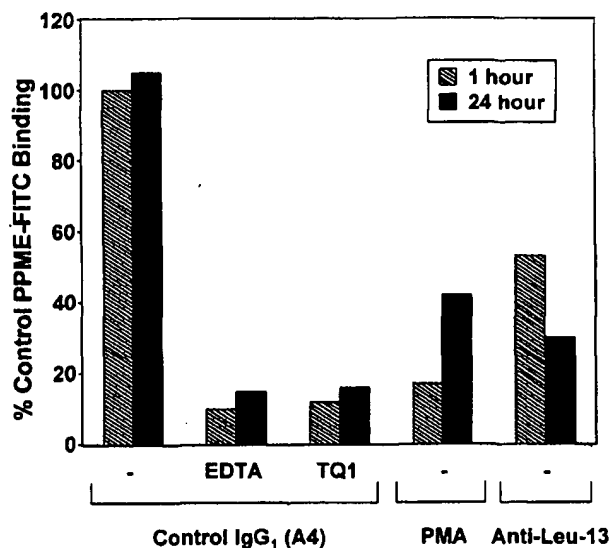


*Leu-13 engagement by mAb inhibits L-selectin-mediated adhesion of human lymphocytes to PPME and to peripheral lymph node HEV*

Studies were performed to determine whether the anti-Leu-13 mAb effects on L-selectin surface density were associated with impaired lymphocyte binding to soluble carbohydrate ligands, i.e., PPME, and to physiologic ligands expressed on peripheral lymph node HEV. Consistent with previous reports (11, 37), calcium-dependent L-selectin-mediated binding of lymphocytes to PPME was inhibited by the cationic chelator, EDTA, and by the L-selectin-specific mAb TQ1 (Fig. 3). Lymphocyte treatment with PMA for 1 h inhibited PPME binding by ~82%; after 24-h continuous exposure to PMA, a slight increase in PPME binding was observed. Short-term, 1-h incubation of lymphocytes with anti-Leu-13 mAb resulted in 50% inhibition of PPME binding, while exposure to anti-Leu-13 mAb for 24 h resulted in a further decrease in PPME binding (~70% inhibition). These data indicate that the level of L-selectin-mediated PPME binding correlates with the effects of anti-Leu-13 mAb and PMA on lymphocyte L-selectin cell surface density.

Studies were also performed to examine the effects of anti-Leu-13 mAb on lymphocyte adhesion to physiologic ligands on vascular endothelial cells *in vitro* under shear conditions, mimicking flow conditions in blood vessels. In the experiment shown in Figure 4A, human PBL were incubated 24 h in medium alone (a) or in the presence of PMA (b), anti-Leu-13 mAb (c), or A4 control mAb (d), and then adhesion to peripheral lymph node HEV was examined in a Stamper-Woodruff adhesion assay (38). A significant level of lymphocyte adhesion to HEV was detected following incubation in medium alone or with A4 control mAb, whereas both PMA and anti-Leu-13 mAb markedly inhibited lymphocyte-HEV interactions.

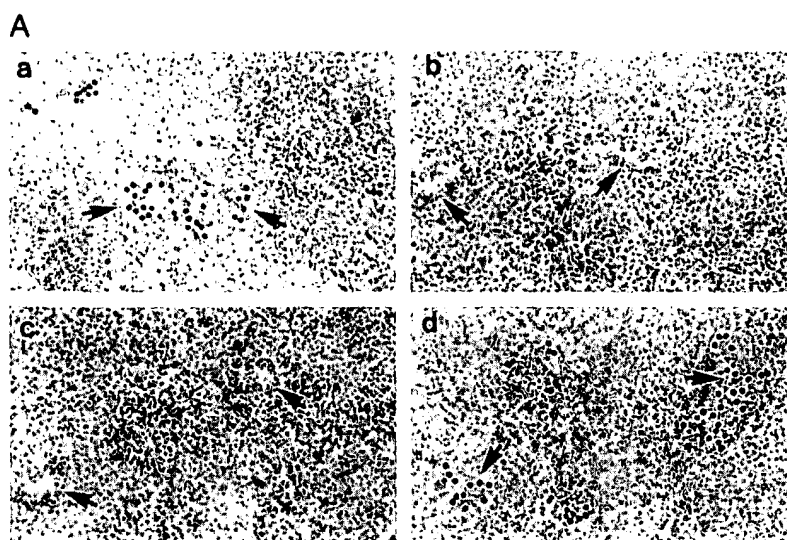
Quantification of lymphocyte binding to HEV following incubation for either 1 or 24 h with PMA or anti-Leu-13 mAb was also performed (Fig. 4B). L-selectin dependence of lymphocyte binding to HEV was confirmed by evidence that the L-selectin-specific mAb DREG56 markedly inhibited adhesion in this assay. Exposure of lymphocytes to PMA for 1 or 24 h consistently inhibited



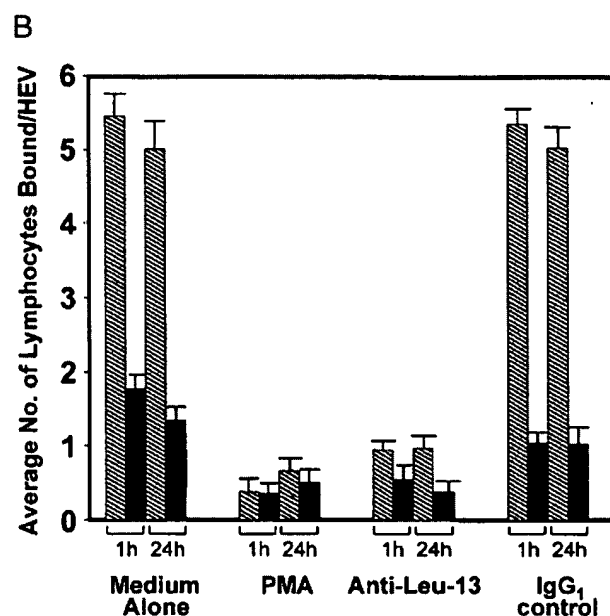
**FIGURE 3.** Leu-13 cross-linking by mAb inhibits L-selectin-dependent binding of lymphocytes to PPME. Human PBL were cultured for 1 or 24 h with A4 Ab (10  $\mu$ g/ml; an isotype-matched control mAb), 50 ng/ml PMA, or 10  $\mu$ g/ml anti-Leu-13 mAb. Cells were then incubated 30 min either in medium alone (—) or with 10 mM EDTA or 10  $\mu$ g/ml TQ1 before the addition of FITC-conjugated PPME, and fluorescence intensity was analyzed by flow cytometry. Data are expressed as a percentage of mean channel fluorescence values of experimental samples relative to untreated controls, and are representative of three independent experiments.

L-selectin-mediated binding of lymphocytes to HEV by greater than 90%, consistent with the low level of L-selectin expression on these cells (Fig. 1). Of particular interest, incubation of lymphocytes for either 1 or 24 h with anti-Leu-13 mAb inhibited L-selectin-dependent adhesion to HEV by approximately 80%, in spite of the fact that L-selectin cell surface density was only partially down-regulated after anti-Leu-13 mAb treatment at these time points, as indicated by the data shown in Figure 1.





**FIGURE 4.** Ligation of Leu-13 by mAb inhibits L-selectin-mediated adhesion of lymphocytes to lymph node HEV. **A**, PBL were cultured 24 h in medium alone (**a**) or with 50 ng/ml PMA (**b**), 10  $\mu$ g/ml anti-Leu-13 mAb (**c**), or 10  $\mu$ g/ml A4 Ab (an isotype-matched control mAb) (**d**). Lymphocyte binding to cryosections of BALB/c peripheral lymph node HEV was assayed as described. Micrographs show representative fields. Darkly stained round cells are the exogenously added lymphocytes that have attached to lymph node sections during the adhesion assay. Arrows indicate HEV structures. **B**, Following culture of lymphocytes under the indicated conditions for 1 or 24 h, cells were then incubated for 30 min with 50  $\mu$ g/ml A4, an isotype-matched control mAb (hatched bars), or with 50  $\mu$ g/ml DREG56 (black bars), and lymphocyte adhesion to lymph node HEV was assessed as described in *Materials and Methods*. Error bars denote SD of three replicate sections. Data are representative of six independent experiments.



#### *Anti-Leu-13 mAb causes sustained down-regulation of L-selectin in the absence of lymphocyte proliferation*

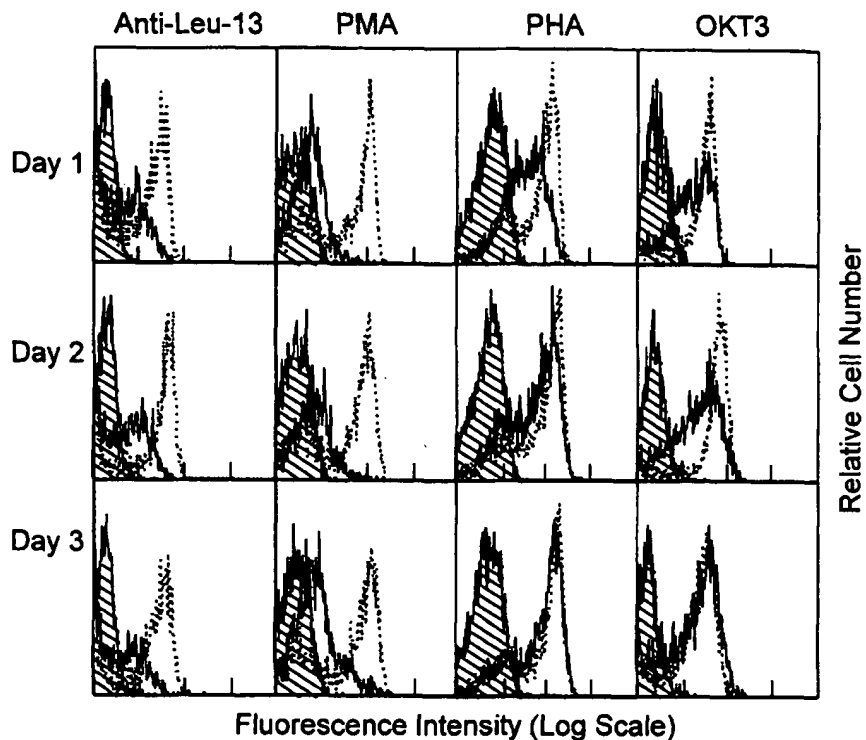
A number of reports have demonstrated that stimulation of lymphocyte proliferation (i.e., by PMA, Ig-cross-linking, and B cell growth factor, IL-2, or anti-CD3 mAb) is associated with transient, partial down-regulation of L-selectin expression (5, 14, 16–21). To determine whether anti-Leu-13 mAb triggered transient or sustained down-regulation of L-selectin, human PBMC were incubated in the absence or presence of anti-Leu-13 mAb, PMA, PHA (a T cell mitogen), or the CD3-specific mAb OKT3, and L-selectin expression by T lymphocytes was determined by multiparameter flow cytometry (Fig. 5). Continuous exposure to anti-Leu-13 mAb markedly down-regulated L-selectin expression by T cells over the first 24 h (Fig. 5). L-selectin levels were maintained at low levels in anti-Leu-13 mAb-treated cultures over a 72-h period (Fig. 5); however, anti-Leu-13 mAb did not markedly increase lymphocyte proliferation (i.e., proliferation index at 72 h was <1.5). While PMA also induced a relatively sustained down-regulation of L-selectin expression, the inhibitory effects of PHA and OKT3 on

L-selectin levels were transient and were observed primarily within the first 24 to 48 h after lymphocyte stimulation, while L-selectin was restored to essentially normal levels by 72 h. Moreover, L-selectin down-regulation observed in T cell populations following incubation with PMA, PHA, or OKT3 mAb was consistently associated with initiation of DNA synthesis (proliferation index at 72 h was 44, 55, and 98, respectively).

#### *Down-modulation of L-selectin surface expression by Leu-13- or L-selectin-specific mAb is dependent on TK activity, but does not involve PKC*

To examine the pathway by which anti-Leu-13 mAb initiates L-selectin down-regulation, studies were performed to compare the effects of kinase inhibitors on PMA- or mAb-induced down-regulation of L-selectin expression. In the experiment shown in Figure 6, human PBL were incubated with staurosporine, a potent inhibitor of PKC (41), or with genistein, a TK-specific inhibitor (42), before the addition of PMA or mAb specific for Leu-13, OKT3, or L-selectin (DREG56). DREG56 was included in this study since

**FIGURE 5.** Anti-Leu-13 mAb causes sustained down-regulation of L-selectin surface expression on T lymphocytes. Human PBMC were cultured for the indicated time intervals in medium alone (dotted histograms) or in the presence of 10  $\mu$ g/ml anti-Leu-13, 50 ng/ml PMA, 1.25  $\mu$ g/ml PHA, or 0.5  $\mu$ g/ml OKT3 (anti-CD3) (solid histograms). L-selectin expression by CD3<sup>+</sup>CD19<sup>-</sup> T cells was determined by multiparameter flow cytometry. Hatched histograms represent the background fluorescence of cells stained with FITC-labeled isotype-matched control mAb. Data are representative of three independent experiments.



mAb-induced L-selectin cross-linking previously has been shown to initiate L-selectin down-modulation in lymphocytes (13, 14, 17).

In control cultures, incubation of PBL with kinase inhibitors or with the nonsignaling Leu-13-specific 13A5 murine IgM mAb did not affect the constitutive level of L-selectin expression (Fig. 6A). Previous studies have demonstrated that Leu-13 ligation by the 13A5 mAb does not initiate intracellular signal transduction pathways in lymphocytes, based on evidence that this mAb fails to induce homotypic adhesion or inhibit lymphocyte proliferation (25). L-selectin down-regulation by PMA, a direct activator of PKC (43), was markedly inhibited by staurosporine, whereas the TK inhibitor genistein did not prevent PMA-induced suppression of L-selectin expression. These data are consistent with the notion that PMA-induced down-regulation of L-selectin surface expression is mediated by activation of the PKC family of serine-threonine kinases and does not involve TK activity. Similar sensitivity to these kinase inhibitors was observed in OKT3-treated cells.

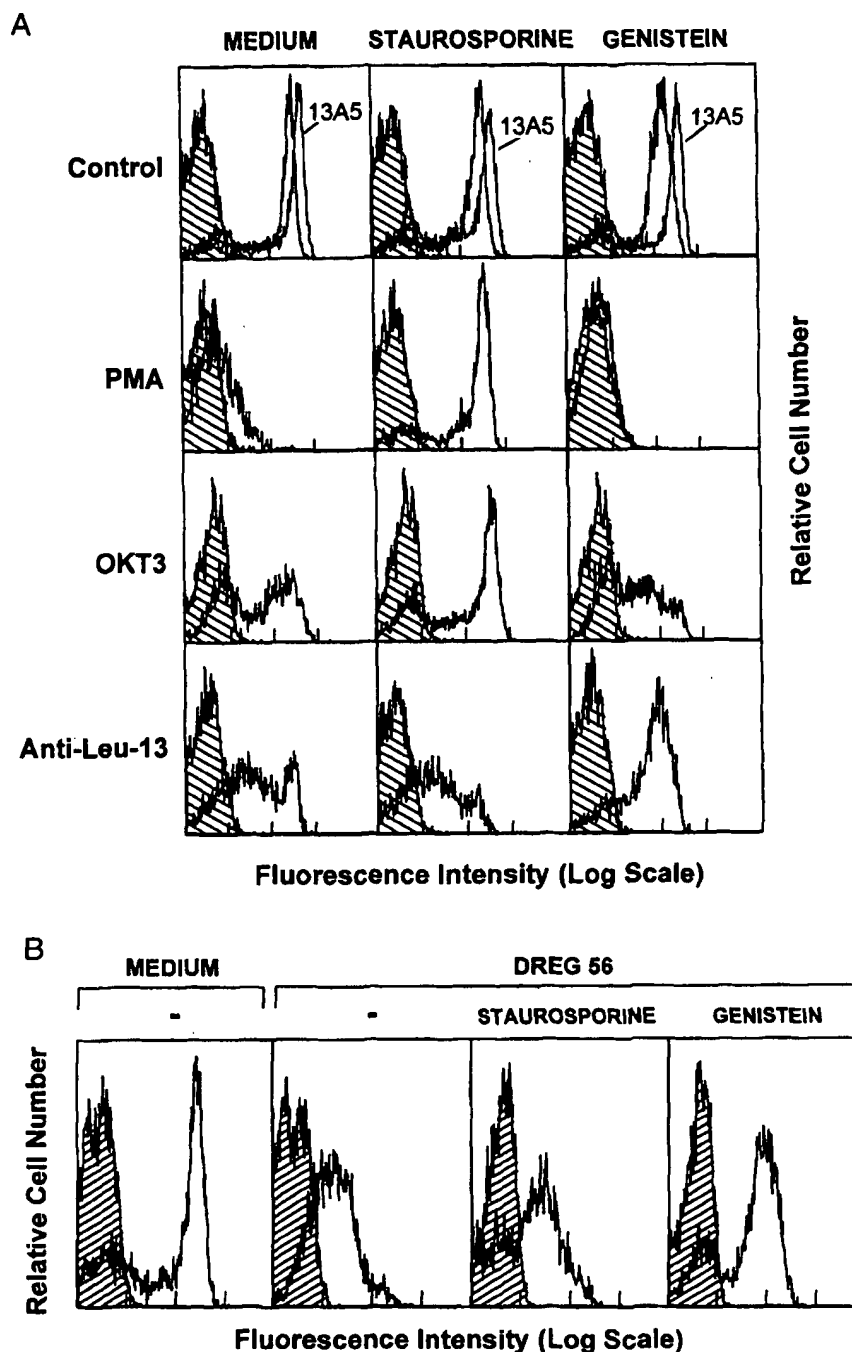
In sharp contrast, anti-Leu-13 mAb-induced suppression of L-selectin surface density was not affected by staurosporine, whereas genistein markedly inhibited the effects of the anti-Leu-13 mAb (Fig. 6A). Identical effects of these kinase inhibitors were observed when L-selectin expression was assessed 1 h after the addition of anti-Leu-13 mAb (data not shown). Moreover, serine-threonine kinase inhibitors, including H7 and the PKC-specific inhibitors calphostin C and chelerythrine, had no effect on L-selectin down-modulation by Leu-13 (not shown). Thus, anti-Leu-13 mAb operates through a novel TK-dependent, PKC-independent pathway to suppress L-selectin surface expression in lymphocytes. Notably, L-selectin ligation by the L-selectin-specific mAb DREG56, modeling cross-linking events that presumably occur during lymphocyte-endothelial cell interactions, markedly down-regulated L-selectin expression through a staurosporine-resistant, genistein-sensitive pathway (Fig. 6B), directly paralleling anti-Leu-13 mAb effects. L-selectin cross-linking by anti-Leu-8 mAb also down-regulated L-selectin expression in a PKC-independent, TK-depen-

dent manner (not shown), although anti-Leu-8 was less efficient than DREG56 in reducing L-selectin surface expression. Taken together, these data demonstrate that L-selectin down-modulation from the lymphocyte cell surface can be triggered through multiple intracellular signaling pathways that differentially depend on PKC or TK activity.

#### *Leu-13 or L-selectin engagement by specific mAb initiates L-selectin shedding*

L-selectin shedding is known to be a major mechanism by which L-selectin density on the plasma membrane is regulated following activation by factors such as PMA (12, 18, 19, 39, 40). Therefore, to determine whether either Leu-13 or L-selectin initiates L-selectin down-modulation from lymphocyte cell surfaces through a shedding mechanism, soluble L-selectin levels in supernatants from PMA- or mAb-treated cultures were determined by ELISA.

As previously described (18, 19), lymphocytes constitutively shed L-selectin from the plasma membrane, as indicated by the accumulation of detectable levels of L-selectin in culture supernatants over a 24-h period (Fig. 7). Incubation of PBL for 1 or 24 h either with the nonsignaling Leu-13-specific 13A5 mAb or isotype-matched murine control mAb (IgG1, IgM) had no effect on the constitutive level of L-selectin shedding. In PMA-treated cultures, high levels of sL-selectin were detected within 1 h of PMA activation, consistent with the nearly complete loss of L-selectin from the cell surface. sL-selectin levels were further elevated 24 h after PMA stimulation, suggesting that newly expressed L-selectin is shed continuously into the medium over this time interval. Ligation of both Leu-13 and L-selectin was also found to initiate L-selectin shedding in a time-dependent manner. The Leu-13-specific signaling mAb (murine IgG1) caused a low, but consistently detectable level of L-selectin shedding during a 1-h incubation, and high soluble L-selectin levels could be detected in culture supernatants after 24 h. L-selectin cross-linking by DREG56 mAb triggered rapid L-selectin shedding, with significantly elevated



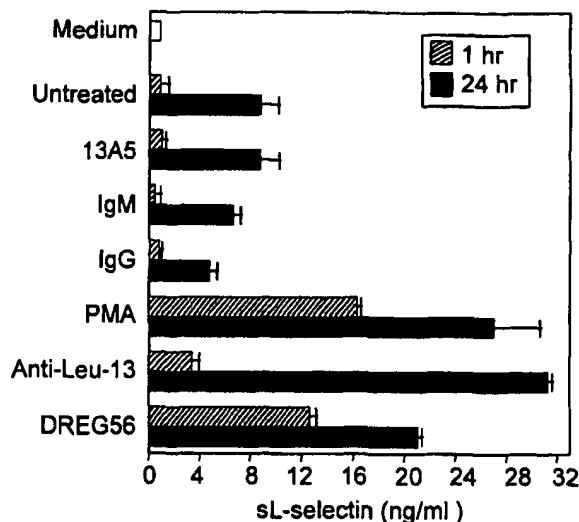
**FIGURE 6.** Leu-13 and L-selectin ligation by mAb induces L-selectin down-regulation through a TK-dependent, PKC-independent mechanism. **A**, PBL were preincubated 30 min in medium alone or with 50 nM staurosporine or 50  $\mu$ g/ml genistein, and then cultured 24 h in medium (control) or with 50 ng/ml PMA, 0.5  $\mu$ g/ml OKT3, 10  $\mu$ g/ml anti-Leu-13 mAb, or 10  $\mu$ g/ml 13A5. **B**, PBL were incubated with medium alone or with 5  $\mu$ g/ml DREG56 mAb for 1 h. L-selectin expression was detected using anti-Leu-8 mAb that bind to distinct epitopes from DREG56. Hatched histograms represent the background fluorescence of cells stained with FITC-labeled isotype-matched control mAb. Data are representative of five independent experiments.

levels detected both 1 and 24 h after mAb-induced L-selectin cross-linking.

## Discussion

These studies provide the first demonstration of a novel level of regulation of L-selectin expression that involves the Leu-13 signal transduction molecule. Specifically, engagement of Leu-13 by murine mAb was shown to markedly inhibit L-selectin surface density on peripheral blood B and T lymphocytes and on malignant Daudi B cells. Moreover, loss of L-selectin surface expression on lymphocytes was attributed to an increase in L-selectin shedding from the plasma membrane. Investigation of the functional conse-

quences of these Leu-13-mediated effects demonstrated that suppression of L-selectin surface density following Leu-13 ligation abrogated L-selectin-dependent binding of lymphocytes to vascular endothelial cells *in vitro*. These results, taken together with previous studies, support the hypothesis that the Leu-13/TAPA-1 signaling complex regulates lymphocyte function through the control of multiple independent adhesion mechanisms including 1) suppression of L-selectin-mediated lymphocyte binding to HEV, as demonstrated in this work, 2) activation of lymphocyte homotypic aggregation responses (22, 25, 27–30, 34), and 3) stimulation of  $\beta_1$  integrin-mediated lymphocyte adhesion to extracellular matrix proteins (44).



**FIGURE 7.** Ligation of Leu-13 by mAb causes L-selectin to be shed from the lymphocyte cell surface. PBL were cultured at  $4 \times 10^6$  cells/ml for 1 or 24 h in medium alone (untreated) or with 50 ng/ml PMA, anti-Leu-13 mAb hybridoma culture supernatant (1/2 final dilution), 10  $\mu$ g/ml 13A5, 5  $\mu$ g/ml DREG56, 10  $\mu$ g/ml A4 Ab (an IgG1 isotype-matched control mAb), or 10  $\mu$ g/ml M104E (an IgM isotype-matched control mAb). sL-selectin levels in culture supernatants were determined by ELISA. The level of sL-selectin detected in medium that did not contain cells is shown. Error bars denote SD of replicate samples; data are representative of four independent experiments.

An unexpected finding was that the partial loss of L-selectin surface expression observed 1 h following ligation of Leu-13 molecules corresponded to maximal inhibition of L-selectin-dependent adhesion of lymphocytes to peripheral lymph node HEV. These results may underscore the requirement for a critical L-selectin density on lymphocyte surface microvilli for avid interactions between L-selectin and its endothelial cell counter-receptors under physiologic conditions (1, 10). Diminution of the overall L-selectin surface density by Leu-13 cross-linking could potentially reduce L-selectin clustering on microvilli to the extent that adherence to HEV can no longer be sustained in the face of high shear forces. An alternative, but not mutually exclusive, interpretation of these data is that Leu-13 ligation perturbs intracellular mechanisms that control L-selectin-binding function, and thereby avidity for endothelial ligands. In this regard, previous studies have suggested that L-selectin adhesion and/or avidity are regulated through physical interactions of the L-selectin cytoplasmic domain with the actin-based cytoskeleton, and by intracellular signals triggered following ligation of the TCR complex (45–47). In recent studies, we have attempted to examine the effects of Leu-13 ligation on L-selectin function in the absence of changes in L-selectin surface density, using genistein to block Leu-13-induced L-selectin down-regulation. However, this TK inhibitor acts directly on lymphocytes to prevent L-selectin-mediated adhesion to HEV, in the absence of anti-Leu-13 mAb (S.S.E., unpublished observations). Thus, the issue of whether the inhibitory effects of anti-Leu-13 mAb on lymphocyte adhesion to HEV reflect changes in L-selectin expression or function awaits the development of a means to evaluate L-selectin avidity at a molecular level.

Several notable differences were observed between the regulation of L-selectin surface density through the Leu-13 molecule and by other lymphocyte signaling mechanisms, suggesting that these molecules operate through distinct pathways to control L-selectin expression. In this regard, L-selectin down-regulation occurred

gradually over a 24-h period in response to anti-Leu-13 mAb, whereas PMA induced rapid L-selectin down-regulation within 1 h of treatment, as previously reported (5, 12, 14, 17–19, 39, 40). Moreover, while continuous exposure to anti-Leu-13 mAb or PMA over a 72-h period resulted in sustained suppression of L-selectin expression, PHA and anti-CD3 (OKT3) mAb effects were transient, with the greatest inhibition observed within the first 24 h of treatment. A unique aspect of anti-Leu-13 mAb-induced suppression of L-selectin expression was that, in contrast to the response to PMA, PHA, or anti-CD3 mAb, Leu-13 ligation was not associated with initiation of lymphocyte activation or proliferation.

The present study further implicates a novel signal transduction pathway in the regulation of L-selectin surface expression that is distinct from the previously well-described PKC-dependent L-selectin shedding mechanism activated in response to PMA (5, 12, 14, 17–19, 39, 40). Specifically, Leu-13 ligation was shown to operate through a genistein-sensitive, TK-dependent pathway to initiate L-selectin down-modulation from the cell surface. The physiologic relevance of this pathway is supported by the finding that direct L-selectin cross-linking by DREG56 or anti-Leu-8 mAb also triggers L-selectin shedding through a TK-dependent mechanism. Moreover, both Leu-13 and L-selectin initiate L-selectin down-regulation through a PKC-independent pathway that is insensitive to staurosporine, a potent inhibitor of PKC activity (41, 48). In a recent report, Stoddart et al. have similarly demonstrated that lymphocyte L-selectin down-modulation induced by L-selectin-specific mAb cross-linking is insensitive to staurosporine (14). Under physiologic conditions, this TK-dependent, PKC-independent pathway initiated either by L-selectin or Leu-13 following interactions with ligands may function to trigger low level loss of L-selectin during initial lymphocyte-endothelial cell interactions. Notably, low level L-selectin shedding recently has been suggested to contribute to the velocity of rolling of lymphocytes along blood vessels (12, 13, 17).

In sharp contrast, evidence presented in this study and in previous reports (5, 14, 18, 21) indicates that PMA and OKT3 mAb down-regulate L-selectin expression via a staurosporine-sensitive, genistein-resistant mechanism. Although genistein, a potent TK inhibitor (42), has been shown to prevent various CD3-induced early activation events in T cells (49), its failure to inhibit either CD3 or PMA-induced L-selectin down-regulation strongly implicates a TK-independent signaling pathway in control of expression of this adhesion molecule. The finding that staurosporine effectively inhibits L-selectin shedding in response to PMA and CD3, known activators of PKC in lymphocytes (43, 50), suggests that a PKC-dependent mechanism is involved, although a role for other serine-threonine kinases is not formally ruled out, since staurosporine is a potent, but relatively nonrestricted inhibitor of protein kinases C, A, and G, and even selected TK (41, 48, 51).

Evidence that Leu-13 regulation of L-selectin expression involves TK activity is consistent with reports that proteins known to associate with this signal transduction molecule are closely linked to TK pathways in lymphocytes. In this regard, homotypic adhesion initiated through Leu-13, TAPA-1, CD19, and CD21 is blocked by inhibitors of TK activity (i.e., genistein, herbimycin A) (28, 30) and by mAb specific for the CD45 tyrosine phosphatase (34). Since CD45 has been implicated specifically in the regulation of the p56/53<sup>lyn</sup> and p56<sup>lck</sup> tyrosine kinases that physically associate with components of the Leu-13/TAPA-1 signal complex, i.e., CD19 in B cells and CD4 or CD8 in T cells (52), it is tempting to speculate that these *src* family TK play an integral role in regulating L-selectin surface density in response to engagement of the Leu-13 complex. Related to these observations, Stibenz et al. (53)

have reported recently that CD45 ligation by selected mAb initiates lymphocyte L-selectin down-regulation via a TK-dependent, PKC-independent mechanism. We have obtained similar results in preliminary studies using anti-CD45 mAb, supporting the hypothesis that Leu-13, L-selectin, and CD45 initiate overlapping signaling cascades to regulate L-selectin surface expression in lymphocytes.

The mechanism by which Leu-13 initiates L-selectin shedding remains to be determined. Recent studies have shown that PMA activates PKC-dependent L-selectin shedding through zinc-dependent metalloproteases (12, 39, 54, 55). Although the specific metalloproteases responsible for L-selectin shedding have not been identified, PKC-dependent pathways have been proposed to cause a change in L-selectin conformation, leading to increased susceptibility of this adhesion molecule to cleavage by constitutively active, membrane-associated metalloproteases. Alternatively, intracellular signals may lead to downstream activation of metalloprotease activity, thereby increasing the rate of L-selectin shedding from the plasma membrane. Studies are currently in progress to determine whether the newly identified TK-dependent pathways initiated by Leu-13, L-selectin, and CD45, on the one hand, and by the PKC signaling pathways triggered through cellular activators such as PMA and OKT3, converge on a common mechanism, i.e., the same class of metalloprotease(s), to initiate L-selectin shedding from lymphocyte surface membranes.

Strategies that specifically induce loss of L-selectin expression by lymphocytes, either through lymphocyte signaling molecules such as Leu-13 or by activating the function of specific metalloproteases, may represent novel approaches toward interfering with L-selectin adhesion events in pathologic disorders. Vascular endothelium within chronically inflamed tissues (i.e., pancreas, thyroid, colon, and synovium) expresses L-selectin ligands identified by the MECA-79 mAb, and thus, is theoretically capable of supporting lymphocyte recruitment via mechanisms similar to those utilized by lymphocytes to traffic through lymph nodes (1, 2, 6, 7). L-selectin has also been implicated in the hematogenous dissemination of malignant cells to lymph nodes in leukemia and lymphoma (3–5). Future therapies based on multiple modes of action, i.e., agents that physically prevent L-selectin-mediated adhesion (mAb, small peptide inhibitors, or carbohydrate inhibitors) together with reagents that inhibit L-selectin expression or function (Leu-13- or metalloprotease-directed agents), are likely to have enhanced efficacy in the treatment of inflammatory disorders and lymphoproliferative malignancies.

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# Monoclonal Antibody to the Interferon-Inducible Protein Leu-13 Triggers Aggregation and Inhibits Proliferation of Leukemic B Cells

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Interferon (IFN)- $\alpha$  inhibits DNA synthesis stimulated by low molecular weight B-cell growth factor (BCGF) in hairy cells in vitro, suggesting that the therapeutic efficacy of IFN- $\alpha$  in hairy cell leukemia (HCL) involves growth inhibition of malignant B cells. Evidence that the 16-Kd cell surface protein Leu-13 mediates an antiproliferative signal in T lymphocytes and is IFN-inducible in endothelial cells prompted us to examine the expression and functional role of this molecule in leukemic B cells. Leu-13 density, determined by flow cytometry, was upregulated in vitro and in vivo by IFN- $\alpha$  on malignant B cells from patients with HCL, chronic lymphocytic leukemia, and prolymphocytic

leukemia. Monoclonal anti-Leu-13 triggered homotypic aggregation of leukemic B cells via an adhesion pathway that was not inhibited by antibodies to leukocyte function associated antigen-1 (LFA-1) or intercellular adhesion molecule-1 (ICAM-1). Moreover, anti-Leu-13 potentiated the inhibitory effects of IFN- $\alpha$  on BCGF-stimulated DNA synthesis, assessed by [ $^3$ H]-thymidine and [ $^3$ H]-deoxyadenosine incorporation into DNA. These results indicate that Leu-13 is part of a novel IFN-inducible signaling pathway which may modify the growth and adhesive properties of leukemic B cells under physiologic or therapeutic conditions.

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**I**NTERFERONS (IFN) are a group of naturally produced cytokines that exert diverse immunomodulatory, antiproliferative, and antitumor effects in vitro and in vivo. IFN- $\alpha$  therapy elicits a variable antileukemic response in related B-cell lymphoproliferative disorders including hairy cell leukemia (HCL) and chronic lymphocytic leukemia (CLL). In this regard, it is now well established that IFN- $\alpha$  is highly effective in the treatment of HCL with response rates of 80% to 90% reported.<sup>1,2</sup> In contrast, IFN- $\alpha$  is generally ineffective in advanced B-cell CLL (B-CLL), with response rates of 18% observed.<sup>3,4</sup> However, recent studies have indicated that IFN may produce clinical benefit in some CLL patients with early stage disease.<sup>5-7</sup>

It has been proposed that the consistent clinical response to IFN- $\alpha$  in HCL results from direct effects on leukemic B-cell growth, differentiation, and recirculation in addition to activation of host immune effector cells.<sup>8,9</sup> In a previous report<sup>10</sup> we have shown that IFN- $\alpha$  inhibits DNA synthesis in HCL lymphocytes stimulated with low molecular weight B-cell growth factor (BCGF) in vitro. Genot et al<sup>11,12</sup> recently confirmed and extended these observations, demonstrating that short-term exposure of hairy cells to IFN- $\alpha$  in vivo inhibits subsequent BCGF-dependent proliferation. These studies have suggested that IFN directly regulates the clonal expansion of malignant B cells in vivo by inhibiting responsiveness to growth factors. IFN- $\alpha$  has also been reported to induce differentiation of normal B lymphocytes, B-HCL, and B-CLL cells in vitro, as assessed by transformation to a plasmacytoid morphology, increased accumulation of intracellular immunoglobulin (Ig), and augmented IgM secretion.<sup>13-15</sup> Furthermore, IFN- $\alpha/\beta$  has been shown to act directly on lymphocytes to increase their uptake and retention in lymph nodes in an animal model.<sup>16</sup> Thus, the rapid reduction in circulating leukemic B cells observed in IFN- $\alpha$ -responsive HCL and CLL patients<sup>1,6</sup> may reflect a direct effect of IFN- $\alpha$  on the recirculation of neoplastic B cells, possibly involving the modulation of homing receptors or adhesion molecules.

It is now clear that although IFN- $\alpha$  binding to high affinity cell surface receptors is required, it is not necessarily sufficient to elicit a biologic response.<sup>5,10,17</sup> These observations have served to emphasize the role of postreceptor binding events in mediating the effects of IFN- $\alpha$ . In this regard, IFN- $\alpha$  induces the expression of a number of gene products

in IFN-sensitive B-lymphoblastoid cell lines and leukemic B cells including major histocompatibility complex (MHC) class I and II molecules, 2'-5'-oligoadenylate synthetase, and several functionally undefined proteins.<sup>9,18,19</sup> Although information is now available regarding the transcriptional regulation of IFN-inducible genes,<sup>20</sup> the role of IFN-inducible proteins in eliciting the immunomodulatory and antileukemic effects of IFN- $\alpha$  remains a critical question.

Recently Jaffe et al<sup>21</sup> reported that the expression of a 16-Kd cell surface protein, designated Leu-13, is inducible by IFN- $\alpha$  and IFN- $\gamma$  on cultured umbilical cord endothelial cells. Previously we showed that Leu-13 is expressed on T and B lymphocytes<sup>22</sup> as well as on vascular endothelial cells examined in frozen tissue sections.<sup>23</sup> Monoclonal antibody (MoAb) to the Leu-13 antigen was found to trigger T-cell aggregation and inhibit T-cell proliferation that is induced by MoAbs to CD3.<sup>22</sup> These observations prompted us to investigate the effects of IFN- $\alpha$  on leukemic B-cell expression of Leu-13 and to determine whether monoclonal anti-Leu-13 modulated responsiveness of malignant B cells to BCGF. In this report we show that IFN- $\alpha$  augments the cell surface density of Leu-13 on normal B lymphocytes and on leukemic B cells isolated from HCL, CLL, and prolymphocytic leukemia (PLL) patients. Our studies further show that

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signaling of leukemic B cells with monoclonal anti-Leu-13 upregulates the function of a novel adhesion pathway and potentiates the inhibitory effects of IFN- $\alpha$  on BCGF-driven proliferation. These data suggest that the IFN-inducible cell surface protein Leu-13 plays an important role in mediating the immunomodulatory and antileukemic effects of IFN- $\alpha$ .

## MATERIALS AND METHODS

**Antibodies.** MoAb anti-Leu-13 is an IgG $\kappa$  protein that has been previously characterized.<sup>22,23</sup> MoAbs specific for CD5 (anti-Leu-1, an IgG $\mu$ ) and CD8 (anti-Leu-2a, an IgG $\kappa$ ) have been previously described.<sup>24,25</sup> MoAbs anti-Leu-14 and anti-Leu-M5, specific for the CD22 and CD11c antigens, respectively, are commercially available (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Anti-Leu-16, an IgG $\kappa$  specific for the CD20 antigen, was obtained commercially (Becton Dickinson). Anti-B $\alpha$ ,<sup>26</sup> an IgG $\mu$  specific for the CD19 antigen, was kindly provided by Dr L. Nadler (Dana-Farber Cancer Institute, Boston, MA). L368,<sup>27</sup> an IgG $\mu$  directed against  $\beta$ -2-microglobulin, was a gift from Dr R. Levy (Stanford University, School of Medicine, Stanford, CA). Murine 4C11 hybridoma protein, an IgG $\kappa$  directed against the phosphorylcholine binding site on the T15 idiotype (kind gift of Dr R. Ward, Roswell Park Memorial Institute, Buffalo, NY), was used as a control antibody in this study. Highly characterized MoAbs specific for cell surface adhesion molecules included: MoAbs specific for the  $\alpha$  chain of leukocyte-function associated (LFA) antigen-1, TS1/22<sup>28</sup> (kindly provided by Dr T.A. Springer, Harvard Medical School, Boston, MA) and 2F12<sup>29</sup> (kindly provided by Dr J. Ritz, Dana-Farber Cancer Institute, Boston, MA); and a MoAb specific for intercellular adhesion molecule-1 (ICAM-1), RR1/1<sup>30</sup> (kind gift from Dr R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). Rabbit polyclonal antibodies to human IFN- $\alpha$  were obtained from Interferon Sciences Inc, New Brunswick, NJ.

**Cell separations.** Enriched populations of leukemic B cells were isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation as described previously.<sup>10</sup> In numerous studies the incorporation of [<sup>3</sup>H]-thymidine by leukemic B cells from patients with white blood cell (WBC) counts greater than  $30 \times 10^3$  cells/mm<sup>3</sup> was not affected by the removal of T lymphocytes and monocytes. Therefore, assessment of cell surface antigen density and the proliferative response of leukemic B cells was determined in most experiments without removal of the small numbers (<5%) of normal leukocytes in our samples. B cells from leukemia patients selected for this study had cell surface phenotypes consistent with malignant B-CLL, B-PLL, and B-HCL (eg, >95% CD19<sup>+</sup>, CD20<sup>+</sup>, and clonal for Ig $\kappa$  or Ig $\lambda$ ). B-HCL cells coexpressed CD11c and CD22, while B-CLL cells were greater than 95% CD5<sup>+</sup>CD19<sup>+</sup>, as previously reported.<sup>24</sup> Enriched populations of peripheral B lymphocytes from normal donors were isolated as previously described.<sup>31</sup> Briefly, peripheral blood mononuclear cells (PBMC) were incubated 45 minutes at 37°C with 5 mg/mL carbonyl iron and iron-ingesting cells were depleted with a magnet (DynaL Inc, Great Neck, NY). Following the subsequent removal of plastic adherent cells, the nonadherent population was rosetted one to two times with 2-aminoethylisothiuronium bromide-treated SRBC (AET-SRBC). The E-rosette negative, B-enriched population was routinely greater than 80% CD19<sup>+</sup> and less than 7% CD3<sup>+</sup>.

**IFN- $\alpha$  treatment of patients.** Patient N.C. with PLL was treated with  $2 \times 10^6$  U/m<sup>2</sup> body surface area of recombinant IFN- $\alpha_2$  (Schering Corp, Bloomfield, NJ) subcutaneously three times a week. Patient B.W. with HCL was treated with  $0.8 \times 10^6$  U IFN- $\alpha_2$ /m<sup>2</sup> body surface area subcutaneously three times a week.

**Flow cytometric analysis of B-cell phenotypes.** The relative expression of the Leu-13 antigen by normal and malignant B cells was determined by indirect immunofluorescence analysis using a FACS 440 (Becton Dickinson, Sunnyvale, CA). Following Fc receptor (FcR) blockade with goat serum for 10 minutes on ice,  $5 \times 10^5$  B cells were incubated with saturating amounts of monoclonal anti-Leu 13 (100  $\mu$ g/mL) for 1 hour, washed once, and incubated for 30 minutes with goat F(ab')<sub>2</sub> antimouse-IgG-fluorescein isothiocyanate (FITC) (Organon Teknika-Cappel, Malvern, PA) at a dilution of 1:30. As a control, murine 4C11 hybridoma protein, an IgG $\kappa$  specific for an irrelevant antigen, was substituted for anti-Leu-13 in the first step. We previously showed that these labeling conditions are required to optimally stain the Leu-13 antigen<sup>22</sup> such that fluorescence intensity is proportional to the number of antibody binding sites per cell. A total of 4,000 events were analyzed and specific fluorescence intensity, reported as mean channel fluorescence based on a linear scale of 0 to 255 channels, was calculated by subtracting control fluorescence values.

**IFN- $\alpha$  receptor analysis.** Analysis of IFN- $\alpha$  binding to malignant B cells was performed in competitive binding assays as previously described.<sup>10,17,31</sup> Briefly, recombinant human IFN- $\alpha$  (rhIFN- $\alpha$ ) (kindly provided by Dr P. Trotta, Schering Corp, Bloomfield, NJ) was iodinated to a final specific activity of 63 mCi/mg. IFN- $\alpha$  receptor expression was determined by incubating  $5 \times 10^6$  cells with 0.5 ng of [<sup>125</sup>I]-IFN- $\alpha$  and increasing amounts of unlabeled ligand for 2 hours at 4°C. Under these conditions maximal binding of [<sup>125</sup>I]-IFN- $\alpha$  occurred within 45 minutes and IFN- $\alpha$  was not internalized or degraded. At the end of the incubation, cell-bound and free [<sup>125</sup>I]-IFN- $\alpha$  were separated by layering samples over 0.2 mL of a 2:1 mixture of di-n-butylphthalate:dinonyl phthalate in microfuge tubes at 4°C. Following centrifugation for 1 min at 10,000g, supernatants were aspirated and microfuge tips containing cell pellets were counted in a Beckman gamma counter with 81% efficiency. Scatchard analysis of the binding data was performed using the LIGAND computer program to facilitate the identification of high affinity binding sites (dissociation constant [ $K_d$ ] of approximately 1 to  $6 \times 10^{-10}$  mol/L).

**Analysis of DNA synthesis in leukemic B cells.** The incorporation of [<sup>3</sup>H]-nucleosides into DNA in malignant B cells was studied by culturing  $2 \times 10^5$  cells/well in 96-well plates in a total volume of 0.2 mL RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS; GIBCO, Grand Island, NY), 0.01% *Staphylococcus aureus* Cowan I (SAC I; Calbiochem, La Jolla, CA), 10% low molecular weight BCGF (vol/vol) (Cellular Products, Buffalo, NY), 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 2 mmol/L L-glutamine as previously described.<sup>10</sup> Consistent with previous reports,<sup>10,32</sup> this BCGF preparation supported both short-term and long-term proliferation of normal and leukemic B cells but did not stimulate the proliferation of T cells. Leukemic B cells failed to incorporate significant amounts of [<sup>3</sup>H]-thymidine in the absence of BCGF and SAC I (<500 cpm/ $2 \times 10^5$  cells). Following incubation of these cultures with rhIFN- $\alpha$  and MoAb to the Leu 13 antigen, cells were pulsed as indicated with either 0.6  $\mu$ mol/L [<sup>3</sup>H]-thymidine (TdR) (6.7 Ci/mmol; New England Nuclear, Boston, MA), 10  $\mu$ mol/L [<sup>3</sup>H]-TdR, or 0.6  $\mu$ mol/L [<sup>3</sup>H]-deoxyadenosine (29 Ci/mmol; Amersham Corp, Arlington Heights, IL) for 4 hours, and DNA was harvested using a PhD cell harvester and counted in a Beckman scintillation counter. For [<sup>3</sup>H]-deoxyadenosine incorporation, a 1-hour incubation at 37°C in 0.1 mol/L NaOH was performed before precipitation with 10% trichloroacetic acid to ensure hydrolysis of labeled RNA, according to the method of Gewart et al.<sup>33</sup> All cultures were performed in triplicate and the standard deviation between replicates was less than 5%. Cells were cultured for 3, 5, 7, and 9 days and maximal incorporation was



routinely detected on day 7. The results reported in this study are based on data obtained following 7 days of culture.

**Quantitation of aggregation.** The number of normal and leukemic B cells in aggregates was quantitated essentially as previously described by Rothlein et al.<sup>30</sup> To block potential FcR sites,  $2 \times 10^5$  B cells were incubated 30 minutes with 50  $\mu\text{g/mL}$  of anti-Leu-2a, an IgG<sub>1</sub> specific for an irrelevant antigen. Cells were then transferred to 96-well plates and incubated in a total volume of 100  $\mu\text{L}$  of 10% FCS/RPMI 1640 supplemented with 1,000 IU/mL IFN- $\alpha$  and 5  $\mu\text{g/mL}$  anti-Leu-13. Ascites containing MoAbs that block aggregation mediated by the interaction of LFA-1 (TS1/22<sup>28</sup> and 2F12<sup>29</sup>) and ICAM-1 (RR1/1<sup>30,34</sup>) were included in cultures at a final dilution of 1:200. In preliminary studies it was established that MoAbs TS1/22 and 2F12 inhibited phorbol myristate acetate (PMA)-induced aggregation of B cells from normal donors to the same extent and, therefore, both antibodies were used throughout this study. After incubation for 18 hours at 37°C, cells were gently resuspended and the number of nonaggregated cells was determined by counting 10  $\mu\text{L}$  of the suspension in a hemocytometer. The total number of cells was determined after the addition of 10 mmol/L EDTA and incubation at 4°C for 30 minutes. Percent aggregation was determined by the following equation:

$$\% \text{ Aggregation} = \left( 1 - \frac{\text{number of nonaggregated cells}}{\text{total number of cells}} \right) \times 100$$

## RESULTS

**IFN- $\alpha$ -induction of Leu-13 antigen in leukemic B cells in vitro and in vivo.** The relative cell surface density of Leu-13 antigen on normal and leukemic B cells was determined by immunofluorescence flow cytometry (Table 1). The initial levels of Leu-13 antigen detected on leukemic B cells measured at zero time were relatively low compared with normal B lymphocytes, but highly variable among different patient samples. Direct comparison of samples incubated 48 hours in the presence or absence of 1,000 IU/mL rIFN- $\alpha$  showed that IFN- $\alpha$  markedly increased Leu-13 antigen density on leukemic B cells isolated from 5 of 5 HCL, 3 of 3 PLL, 11 of 13 early stage (stage 0 through II) CLL, and 4 of 4 advanced stage (stage III) CLL patients. IFN- $\alpha$  similarly upregulated Leu-13 expression on peripheral B lymphocytes isolated from normal donors. IFN- $\alpha$  failed to upregulate Leu-13 antigen density in 2 of 13 experiments involving B cells from early stage CLL patients (stage 0 CLL patient J.B. and stage I CLL patient R.R.), possibly reflecting a defective in vitro response to IFN- $\alpha$  in a subset of B-CLL patients. Analysis of leukemic B cells from five patients indicated that equivalent concentrations of rIFN- $\gamma$  (Scher-

Table 1. IFN- $\alpha$  Induction of Leu-13 Antigen on Normal and Leukemic B Cells in Vitro

Disease	Stage	Patient	Leu-13 Ag Expression* (specific fluorescence intensity)			IFN- $\alpha$ Receptor†	
			0 h	48 h	48 h + IFN- $\alpha$	R/C	K <sub>d</sub> ( $\times 10^{-10}$ mol/L)
CLL	0	J.B.	ND	85.0	70.1	ND	ND
		J.G.	4.3	43.0	124.4 (70.0)	1,680	2.8
		J.I.	20.6	26.0	121.4 (70.4)	ND	ND
		W.K.	ND	64.3	124.0	425	2.1
		C.T.	7.1	25.9	83.7	380	1.8
	I	E.B.	10.4	33.2	113.0 (68.0)	707	5.5
		E.C.	14.0	16.8	141.7	3,431	17.4
		B.F.	0.0	44.1	119.1	1,100	4.3
		R.R.	6.4	23.9	16.3	223	1.6
		B.W.	0.0	25.9	61.8	539	3.5
	II	A.B.	5.0	12.4	138.9	2,091	3.1
		W.F.	15.3	54.8	175.0	1,066	2.0
		M.P.	19.2	94.3	146.9 (99.0)	1,628	1.9
	III	R.G.	2.3	23.3	109.2	945	2.5
		H.L.	7.0	12.8	77.0	<50	—
		I.P.	0.9	2.1	31.7	949	7.9
		F.W.	17.8	41.0	96.1	2,285	4.9
		N.C.	1.9	13.0	83.1	538	1.0
PLL		G.P.	0.0	9.9	40.5	1,018	6.5
		C.P.	7.6	7.2	63.3	5,898	12.3
		H.K.	3.8	13.7	116.0 (42.0)	1,707	7.5
HCL		T.L.	ND	43.1	144.3	544	4.4
		E.R.	ND	12.5	47.3	330	2.4
		B.W.	8.3	1.1	118.5	2,259	4.7
		S.Z.	ND	16.9	58.4	1,000	2.1
		—	26.0	32.0	118.0	ND	ND
Normal donor‡			44.0	52.0	108.0	ND	ND

Values in parentheses represent specific linear fluorescence of Leu-13 following incubation of cells for 48 hours with 1,000 IU/mL IFN- $\gamma$  (Schering Corp).

Abbreviation: ND, not determined.

\*Leu-13 antigen density was assessed by flow cytometry at 0 hours and following culture for 48 hours  $\pm$  1,000 IU/mL IFN- $\alpha$ .

†The limit of detection of high affinity IFN- $\alpha$  receptors was greater than 50 receptors/cell (R/C).

‡An enriched population of peripheral blood B cells was isolated from normal donors ( $>80\%$  CD19 $^{+}$  and  $<7\%$  CD3 $^{+}$ ).

Table 2. IFN- $\alpha$  Induction of Leu-13 Antigen In Vivo

		Leu-13 Ag Expression (specific linear fluorescence)						
		Pre-IFN- $\alpha$ Treatment*		Days Post-IFN- $\alpha$ Treatment*				
Diagnosis	Patient			1	2	14	21	28
PLL	N.C.	1.9		44.9	19.2	ND	ND	21.0
HCL	B.W.	10.1		52.9	57.2	88.4	136.6	103.3

Abbreviation: ND, not determined.

\*Peripheral WBC counts were greater than  $44 \times 10^3/\text{mm}^3$  and percent lymphocytes was greater than 95% at these time points.

ing Corp) were also capable of increasing Leu-13 expression, although less efficiently than IFN- $\alpha$ . The induction of Leu-13 antigen by IFN- $\alpha$  shown by flow cytometric analysis in Table 1 was confirmed in selected patient samples by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, as previously described for endothelial cells<sup>21</sup> (data not shown).

The constitutive expression of cell surface IFN- $\alpha$  receptors on leukemic B cells was determined in competitive IFN- $\alpha$  binding studies (Table 1). A significant number of specific high affinity receptors for IFN- $\alpha$  were detected in a majority of patient samples examined. However, in these studies the number of high affinity IFN- $\alpha$  receptors expressed on leukemic B cells did not correlate ( $P > .05$ ) with the level of upregulation of the Leu-13 antigen. For example, similar increases in Leu-13 density were detected in B-HCL cells from patients T.L. and B.W. expressing 544 and 2,259 receptors/cell, respectively.

To determine if the Leu-13 antigen was modulated by IFN- $\alpha$  in vivo, the expression of Leu-13 was monitored in two patients undergoing IFN- $\alpha$  therapy. Leu-13 antigen density on leukemic B cells was evaluated by immunofluorescence analysis immediately before therapy and up to 28 days following the initiation of IFN- $\alpha$  treatment. The data shown in Table 2 indicate that the expression of the Leu-13 molecule was augmented in circulating leukemic B cells during the course of IFN- $\alpha$  therapy. Although the relationship between the level of induction of Leu-13 in vivo and clinical responsiveness to IFN- $\alpha$  was not determined in this limited study, these data show that modulation of Leu-13 by IFN- $\alpha$  is not restricted to in vitro culture conditions.

**Dose-dependence, cycloheximide sensitivity, and kinetics of Leu-13 induction by IFN- $\alpha$ .** B cells from two HCL patients and one CLL patient were incubated with increasing concentrations of rIFN- $\alpha$  for 48 hours before the assessment of the Leu-13 antigen density by immunofluorescence analysis.

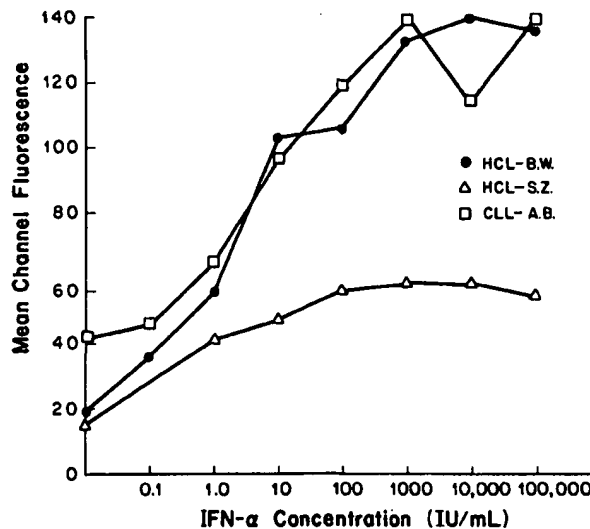


Fig 1. Dose-dependent induction of Leu-13 by IFN- $\alpha$ . Leukemic B cells were incubated with increasing concentrations of rIFN- $\alpha$  for 48 hours. The relative expression of Leu-13 antigen was determined by indirect immunofluorescence analysis.

sis. The results shown in Fig 1 demonstrate that the Leu-13 antigen was markedly increased by IFN- $\alpha$  in a dose-dependent manner in vitro. Significantly, induction of the Leu-13 antigen occurred at relatively low doses of IFN- $\alpha$  ranging from 0.1 to 100 IU/mL, demonstrating that this antigen can be regulated under both physiologic and pharmacologic conditions.

To determine if protein synthesis was required for the induction of Leu-13 by IFN- $\alpha$ , leukemic B cells from CLL patient N.W. were incubated with and without 50  $\mu\text{g}/\text{mL}$  cycloheximide for 2 hours before induction with 1,000 IU/mL IFN- $\alpha$  (Fig 2). In the positive control, exposure of B-CLL cells to IFN- $\alpha$  alone for 18 hours significantly increased the Leu-13 cell surface antigen density (Fig 2b; specific linear fluorescence = 118.2) relative to untreated cells (Fig 2a; specific linear fluorescence = 5.6). Consistent with the report of Jaffe et al<sup>21</sup> on endothelial cells, cycloheximide pretreatment of leukemic B cells abrogated the induction of the Leu-13 antigen (Fig 2c; specific fluorescence intensity = 11.1), suggesting that upregulation of cell surface Leu-13 requires new protein synthesis rather than mobilization from intracellular precursor pools.

Isolated B cells from two HCL patients were incubated with 1,000 IU/mL IFN- $\alpha$  in vitro for the indicated period of

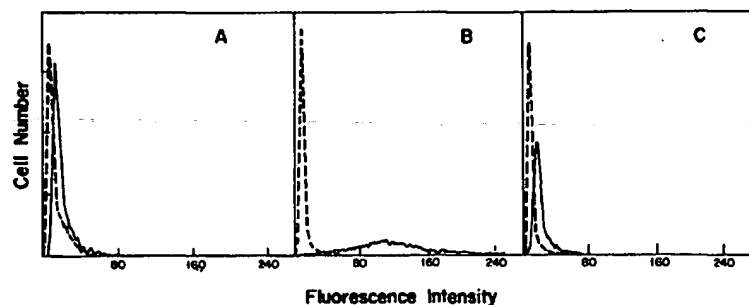


Fig 2. Cycloheximide inhibits Leu-13 induction by IFN- $\alpha$ . B-CLL cells were cultured 18 hours either in medium alone (A) or in the presence of 1,000 IU/mL IFN- $\alpha$  (B) before indirect immunofluorescence analysis. Relative immunofluorescence intensity following staining with second antibody control (---) and anti-Leu-13 (—) are shown. In (C), leukemic B cells were preincubated 2 hours with 50  $\mu\text{g}/\text{mL}$  cycloheximide before the addition of IFN- $\alpha$ . Cell viability following cycloheximide treatment was greater than 90%.

time to determine the kinetics of Leu-13 antigen induction (Fig 3). IFN- $\alpha$  rapidly induced cell surface Leu-13 antigen on leukemic B cells with increased antigen density detectable as early as 8 hours on B-HCL cells from patient B.W. (Fig 3). Increased expression of the Leu-13 antigen was maintained for greater than 5 days in these experiments.

*IFN- $\alpha$  augments the capacity of anti-Leu-13 to trigger homotypic aggregation of leukemic B cells.* Leukemic B cells from CLL and HCL patients reportedly express low levels of LFA antigen-1 and ICAM-1, which mediate adhesions between lymphocytes triggered by activators of protein kinase C (PKC).<sup>34,35</sup> This observation may account for the fact that CLL cells have a reduced capacity to aggregate in the presence of PKC-activators, including PMA.<sup>35</sup> Because we have previously shown that anti-Leu-13 induces peripheral blood T cells to aggregate *in vitro*<sup>22</sup> it was of interest to determine the capacity of anti-Leu-13 and IFN- $\alpha$  to modulate the adhesiveness of leukemic B cells. A representative experiment is shown in Fig 4. Following the blocking of potential FcR sites with anti-Leu-2a, an IgG $\kappa$  specific for an irrelevant antigen, leukemic B cells isolated from an HCL patient (S.Z.) were cultured at a final concentration of  $10^6$  cells/mL for 18 hours at 37°C in the presence of (a) medium, (b) 1,000 IU/mL IFN- $\alpha$ , (c) 5  $\mu$ g/mL anti-Leu-13, or (d) 1,000 IU/mL IFN- $\alpha$  and 5  $\mu$ g/mL anti-Leu-13. While medium alone (Fig 4a) or 1,000 IU/mL IFN- $\alpha$  (Fig 4b) did not directly induce aggregation, anti-Leu-13 triggered the formation of small aggregates (Fig 4c). Aggregation of leukemic B cells was markedly increased by coculture with IFN- $\alpha$  and anti-Leu-13 (Fig 4d). Aggregation triggered by IFN- $\alpha$  and anti-Leu-13 occurred rapidly (3 to 8 hours) in parallel with the induction of the Leu-13 antigen. In repeated experiments, similar results were obtained using lower doses of IFN- $\alpha$  (eg, 10 and 100 IU/mL) and B-CLL, HCL, and PLL cells, which generally expressed less than 25% the amount of LFA-1 $\alpha$  subunit detected on normal peripheral blood B cells (data not shown). Leukemic B-cell aggregation

did not occur in the presence of 10 mmol/L EDTA or following culture at 4°C. MoAbs directed against several other cell surface molecules expressed on B-CLL cells at similar densities as IFN-induced Leu-13, including CD5 (anti-Leu-1, mean channel fluorescence  $\pm$  SD =  $89 \pm 13$ ,  $n = 10$ ), CD19 (anti-B4, mean channel fluorescence  $\pm$  SD =  $116 \pm 19$ ,  $n = 10$ ), and  $\beta$ -2-microglobulin (L368, mean channel fluorescence  $\pm$  SD =  $110 \pm 12$ ,  $n = 4$ ), did not induce B-CLL cellular adhesion (<10% cells in aggregates, data not shown). MoAbs anti-B $_4$  and L368 further serve as controls for possible involvement of FcR-mediated cellular cross-linking because they have the same isotype as anti-Leu-13.

To determine if the aggregation triggered by IFN- $\alpha$  and anti-Leu-13 resulted from increased intercellular LFA-1-ICAM-1 binding, the number of normal and leukemic B cells in aggregates was quantitated following incubation in the presence of MoAbs that specifically block adhesion via this pathway (Table 3). IFN- $\alpha$  and anti-Leu-13 induced cellular adhesion was stable, allowing quantitation of the degree of aggregation, and was inhibited by the inclusion of rabbit polyclonal antibody to human IFN- $\alpha$ . The results shown in Table 3 indicate that PMA induces significantly less aggregation of leukemic B cells (22% aggregation, SD = 11%,  $n = 6$ ; legend) as compared with normal donor B lymphocytes (71% aggregation) and confirm the data reported by Inghirami et al.<sup>35</sup> Aggregation of normal donor B cells induced by PMA was inhibited 65% and 58%, respectively, by MoAbs to LFA-1 $\alpha$  (TS1/22 and 2F12) and ICAM-1 (RR1/1) (Table 3), as previously described.<sup>28,34</sup> In contrast, IFN- $\alpha$  and anti-Leu-13 triggered similar aggregation of normal B lymphocytes (80% aggregation) and leukemic B cells (84% aggregation, SD = 6%,  $n = 6$ ) while MoAbs TS1/22, 2F12, and RR1/1 did not significantly inhibit aggregate formation. Thus, these studies indicate that the aggregation signal provided by IFN- $\alpha$  and anti-Leu-13

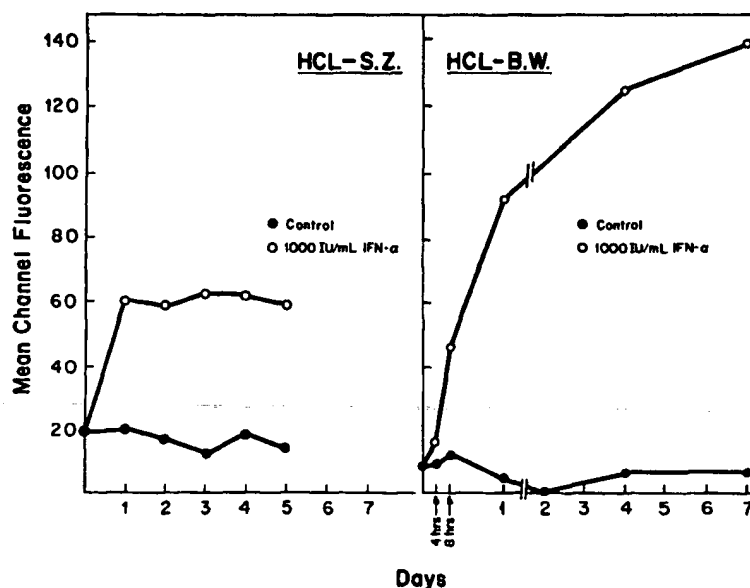


Fig 3. Kinetics of Leu-13 antigen induction by IFN- $\alpha$ . Leukemic B cells were cultured for the indicated time periods in either culture medium (●) or 1,000 IU/mL IFN- $\alpha$  (○). Leu-13 antigen expression was assessed by indirect immunofluorescence analysis.

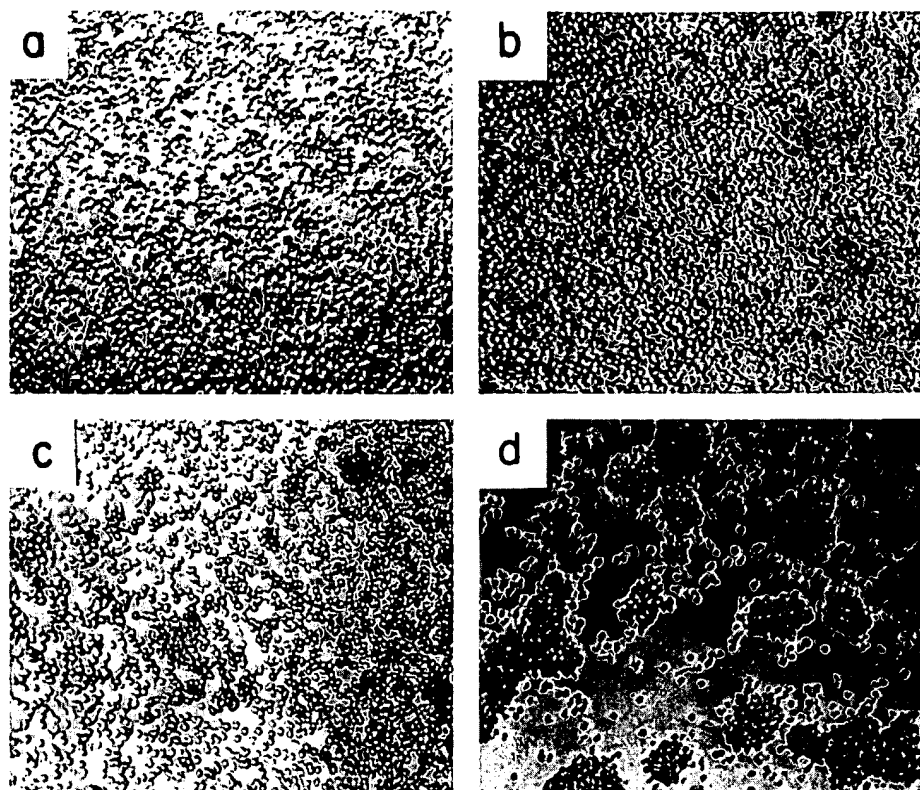


Fig 4. The combination of IFN- $\alpha$  and anti-Leu-13 triggers homotypic aggregation of leukemic B cells. B-HCL cells (S.Z.) were cultured at a final concentration of  $10^6$  cells/mL for 18 hours at 37°C in the presence of culture media (a); 1,000 IU/mL IFN- $\alpha$  (b); 5  $\mu$ g/mL anti-Leu-13 (c); or 1,000 IU/mL IFN- $\alpha$  and 5  $\mu$ g/mL anti-Leu-13 (d).

involves an adhesion pathway distinct from a LFA-1-ICAM-1-dependent interaction.

**Effect of IFN- $\alpha$  and anti-Leu-13 on BCGF-induced [ $^3$ H]-nucleoside incorporation.** IFN- $\alpha$  has previously been shown to inhibit [ $^3$ H]-thymidine incorporation in the Daudi B-lymphoblastoid cell line without altering overall DNA synthesis by decreasing thymidine transport and thymidine kinase activity.<sup>33</sup> These studies have raised the concern that inhibition of DNA labeling with exogenous [ $^3$ H]-thymidine in leukemic B cells may not reflect changes in DNA synthesis after IFN treatment. Therefore, leukemic B cells isolated from a HCL patient (B.W.) were cultured for 7 days with

SAC I/BCGF and increasing concentrations of IFN- $\alpha$  in the presence or absence of 5  $\mu$ g/mL anti-Leu-13 and then pulsed with either 0.6  $\mu$ mol/L [ $^3$ H]-thymidine, 10  $\mu$ mol/L [ $^3$ H]-thymidine, or 0.6  $\mu$ mol/L [ $^3$ H]-deoxyadenosine. The data shown in Fig 5 indicate that [ $^3$ H]-thymidine incorporation assessed following incubation with 0.6  $\mu$ mol/L [ $^3$ H]-thymidine reflects changes in DNA synthesis in leukemic cells because (1) comparable inhibition of [ $^3$ H]-thymidine incorporation was detected at 0.6  $\mu$ mol/L thymidine and at higher thymidine concentrations of 10  $\mu$ mol/L, at which uptake reportedly is not transport dependent,<sup>33</sup> and (2) incorporation of another nucleoside precursor, [ $^3$ H]-deoxy-

Table 3. Anti-LFA-1 and Anti-ICAM-1 Fail to Inhibit B-Cell Aggregation Induced by IFN- $\alpha$  and Anti-Leu-13

Diagnosis	Patient	Stimulation	% Aggregation*		
			Control	Anti-LFA-1 $\alpha$	Anti-ICAM-1
Normal donor†	—	PMA	71	18	30
		IFN- $\alpha$ /anti-Leu-13	80	76	81
CLL (stage 0)	J.I.	IFN- $\alpha$ /anti-Leu-13	75	75	82
CLL (stage 0)	C.T.	IFN- $\alpha$ /anti-Leu-13	93	90	89
CLL (stage I)	B.F.	IFN- $\alpha$ /anti-Leu-13	86 (25)	79	78
CLL (stage II)	M.P.	IFN- $\alpha$ /anti-Leu-13	92 (6)	95	86
CLL (stage III)	M.G.	IFN- $\alpha$ /anti-Leu-13	80	72	74
HCL	H.K.	IFN- $\alpha$ /anti-Leu-13	79	87	86

Percent aggregation induced by 5 ng/mL PMA was as follows: J.I. (31), C.T. (10), B.F. (20), M.P. (21), M.G. (10), and H.K. (42). Values in parentheses represent the percent aggregation that occurred in the presence of 1,000 IU/mL IFN- $\alpha$ , 5  $\mu$ g/mL anti-Leu-13 and 1,000 neutralizing units of rabbit polyclonal antihuman IFN- $\alpha$ .

\*The number of cells in aggregates (% aggregation) was quantitated 18 hours after the simultaneous addition of MoAbs to cell surface adhesion molecules and either PMA (5 ng/mL) or IFN- $\alpha$ /anti-Leu-13. Less than 5% of the cells aggregated in the absence of either PMA or IFN- $\alpha$ /anti-Leu-13. Data represent the average of duplicate wells; standard deviation between replicates was <5%.

†An enriched population of peripheral blood B cells was isolated from a normal donor (>80% CD19<sup>+</sup> and <7% CD3<sup>+</sup>).

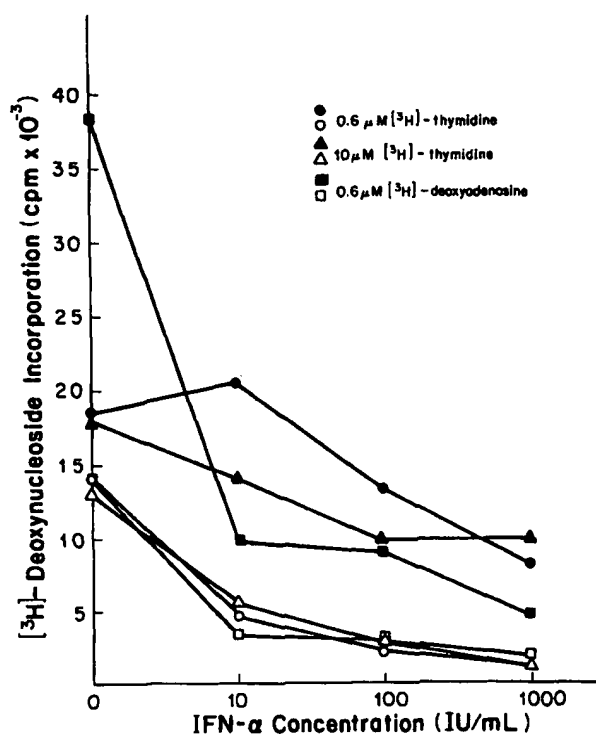


Fig 5.  $[^3\text{H}]$ -nucleoside incorporation in leukemic B cells. B-HCL cells (B.W.) were cultured in 10% BCGF/0.01% SAC I with increasing concentrations of IFN- $\alpha$  in the absence (closed symbols) or presence (open symbols) of 5  $\mu\text{g}/\text{mL}$  anti-Leu-13. On day 7 cells were pulsed 4 hours with 0.6  $\mu\text{mol}/\text{L}$   $[^3\text{H}]$ -thymidine (●,○), 10  $\mu\text{mol}/\text{L}$   $[^3\text{H}]$ -thymidine (▲,△), or 0.6  $\mu\text{mol}/\text{L}$   $[^3\text{H}]$ -deoxyadenosine (■,□).

adenosine, was similarly inhibited by IFN- $\alpha$  and anti-Leu-13. Similar results were obtained using leukemic B cells from other HCL and CLL patients (data not shown).

Based on these studies, the effect of IFN- $\alpha$  and anti-Leu-13 on SAC I/BCGF-induced thymidine incorporation in leukemic cells was further evaluated. Representative data from experiments involving 20 CLL, 3 PLL, and 5 HCL patients are shown in Fig 6. Consistent with our previous report,<sup>10</sup> IFN- $\alpha$  inhibited DNA synthesis in B-HCL cells to a variable extent. Culture of leukemic B cells with anti-Leu-13 in the absence of IFN- $\alpha$  consistently decreased  $[^3\text{H}]$ -thymidine incorporation, which may partially reflect the moderate increase in Leu-13 antigen observed during culture in the absence of IFN- $\alpha$  (Table 1). However, the most significant inhibition of DNA synthesis in B-CLL, HCL, and PLL cells was detected following culture with the combination of IFN- $\alpha$  and anti-Leu-13. In two control experiments shown in Fig 6, equivalent concentrations of anti-Leu-1, an IgG<sub>2a</sub>, did not significantly inhibit thymidine incorporation in CD5-positive B-CLL cells (specific fluorescence intensity of anti-Leu-1 on E.C. and R.G. B-CLL cells was 85.0 and 98.0, respectively), supporting the conclusion that antibody-dependent cytotoxicity mechanisms are not involved in this system. Of particular interest, anti-Leu-13 inhibited DNA synthesis in leukemic B cells from both early and late stage CLL patients (stage 0 through II v stage III) as well as in B cells that were not highly responsive to the antiproliferative effects of IFN- $\alpha$  in vitro (eg, CLL-W.F., PLL-G.P., HCL-S.Z.). However, it is noteworthy that IFN- $\alpha$  was capable of increasing the Leu-13 antigen density on these cells (Table 1). These results indicate that signaling of leukemic B cells with a MoAb to the IFN-inducible Leu-13 antigen in vitro

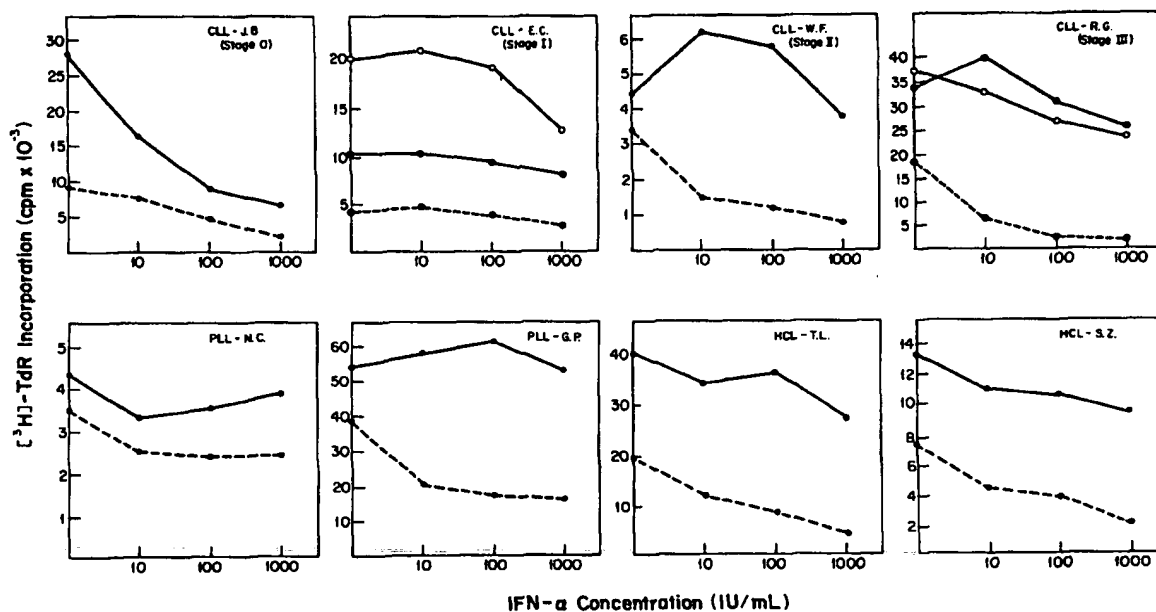


Fig 6. IFN- $\alpha$  and anti-Leu-13 inhibit  $[^3\text{H}]$ -thymidine incorporation in leukemic B cells. Leukemic B cells were cultured in 10% BCGF/0.01% SAC I in combination with increasing concentrations of IFN- $\alpha$  alone (●—●) or in the presence of 5  $\mu\text{g}/\text{mL}$  anti-Leu-13 (○—○). In two experiments (CLL-E.C., CLL-R.G.) 5  $\mu\text{g}/\text{mL}$  of anti-Leu-1 was included in cultures as a control (○—○). On day 7 cells were pulsed 4 hours with 0.6  $\mu\text{mol}/\text{L}$   $[^3\text{H}]$ -thymidine.

can trigger an antiproliferative signal in cells that are either sensitive or resistant to the direct growth inhibitory effects of IFN- $\alpha$ .

### DISCUSSION

Despite intensive investigation, the mechanisms of action of IFN- $\alpha$  in eliciting an antitumor response in B-cell leukemia are currently not well understood. In the present study we have shown that IFN- $\alpha$  acts both in vitro and in vivo to upregulate the cell surface density of Leu-13 on leukemic B cells in a majority of patients with CLL, PLL, and HCL. Induction of Leu-13 antigen by IFN- $\alpha$  in vitro occurred rapidly in a dose-dependent manner and required new protein synthesis. It was of interest that IFN- $\gamma$ , which binds to cell surface receptors distinct from the IFN- $\alpha$  receptor, was less effective at increasing the final Leu-13 density on leukemic B cells. Similar levels of induction of Leu-13 by IFN- $\alpha$  were observed in these malignant B cells despite reported differential response rates to IFN- $\alpha$  therapy in these patient groups.<sup>1,3,7</sup> Furthermore, Scatchard analysis of <sup>125</sup>I-IFN- $\alpha$  binding to leukemic B cells did not show a significant correlation ( $P > .05$ ) between IFN- $\alpha$  cell surface receptor expression and either patient diagnosis or the extent of upregulation of Leu-13 antigen. These results are consistent with recent reports showing that the level of IFN receptor expression on HCL and CLL cells did not correlate either with the clinical response to IFN- $\alpha$ <sup>5,17</sup> or the degree of induction by IFN of the cytoplasmic protein 2'-5'-oligoadenylate synthetase in vitro.<sup>18,36</sup> Taken together, these studies indicate that the interaction of IFN- $\alpha$  with a limited number of high affinity cell surface receptors is sufficient to signal the induction of proteins including Leu-13.

Our studies show that signaling of the IFN-inducible cell surface protein Leu-13 with a specific MoAb triggers homotypic aggregation of normal and leukemic B cells. Several lines of evidence support the conclusion that aggregation mediated by anti-Leu-13, an IgG $\kappa$ , results from an active process rather than trivial cross-linking of cell surface antigens on adjacent cells: (1) aggregation occurs at 37°C but not at 4°C; (2) aggregate formation was not inhibited by preblocking FcR sites with anti-Leu-2a, a murine IgG $\kappa$ ; and (3) aggregation was not mediated by MoAbs to major leukemic B-cell surface antigens expressed at similar levels as IFN- $\alpha$ -induced Leu-13 (eg, CD5, CD19, and  $\beta$ -2-microglobulin). Taken together these studies indicate that homotypic aggregation triggered by anti-Leu-13 is not the result of either passive agglutination or FcR dependent pathways.

It was of interest in the present study to determine if the homotypic adhesion triggered by IFN- $\alpha$  and anti-Leu-13 involves the interaction of LFA-1 and ICAM-1 because these well-characterized molecules are known to mediate aggregation of B lymphocytes.<sup>34,37</sup> Numerous studies have clearly indicated that these adhesion molecules are important in mediating cell-cell interactions involved in the immune response and lymphocyte trafficking.<sup>37,38</sup> Additionally, a recent report<sup>39</sup> has suggested that the expression of LFA-1 on malignant cells is also required to initiate an antitumor

response to lymphoid malignancies. Results obtained in the present study confirm previous reports<sup>35,40,41</sup> showing that leukemic B cells from CLL and HCL patients express low levels of cell surface LFA-1 and aggregate poorly when stimulated with phorbol esters. In contrast, homotypic aggregation of leukemic B cells was triggered consistently by the combination of IFN- $\alpha$  and anti-Leu-13 although the cell surface density of LFA-1 and ICAM-1 was not altered (data not shown). Moreover, aggregation of normal and leukemic B cells triggered by IFN- $\alpha$  and anti-Leu-13 was not inhibited by MoAbs to either LFA-1 or ICAM-1. Because MoAb to the  $\alpha$  subunit of LFA-1 (TS1/22) blocks aggregation mediated by the interaction of LFA-1 with both ICAM-1 and ICAM-2,<sup>42</sup> our data indicate that IFN- $\alpha$  and anti-Leu-13 upregulate a previously undescribed leukocyte adhesion pathway in B lymphocytes. Based on these results, it is tempting to speculate that the decrease in lymphocyte recirculation observed in HCL and CLL patients during IFN therapy<sup>1,2,6</sup> and in animals treated with IFN<sup>16,43</sup> involves signaling of this adhesion pathway at the lymphocyte level.

Our studies further demonstrate that signaling of leukemic B cells with monoclonal anti-Leu-13 potentiates the inhibitory effects of IFN- $\alpha$  on BCGF-stimulated DNA synthesis. This finding is of interest because BCGF has been proposed to have a central role in the clonal expansion of malignant B cells in HCL. In this regard, Ford et al<sup>44</sup> have shown that hairy cells synthesize and secrete an autostimulatory factor that is functionally and biochemically similar to the low molecular weight form of BCGF produced by T cells. We<sup>10</sup> and other investigators<sup>11,12,45</sup> have shown that malignant B cells from HCL patients are highly sensitive in vitro to the proliferative signal provided by exogenous low molecular weight BCGF, and that exposure of leukemic cells to IFN- $\alpha$  either in vitro or in vivo inhibits this proliferative response. These observations were confirmed in the present study using methods which distinguish between DNA synthesis *per se* and incorporation of [<sup>3</sup>H]-thymidine into DNA. Furthermore, in this system the proliferative response of leukemic B cells to BCGF and SAC I in vitro was shown to be significantly suppressed by the combination of IFN- $\alpha$  and anti-Leu-13. Thus, the IFN-inducible molecule Leu-13 appears to be a potent inhibitor of responsiveness to BCGF in B-HCL cells.

In CLL, the role of BCGF in the expansion of neoplastic cells has not been fully elucidated. The proliferative response of B-CLL cells to BCGF is highly heterogeneous compared with B-HCL cells,<sup>46,47</sup> and B-CLL cells reportedly express low levels of BCGF receptors.<sup>48,49</sup> In the present study we showed that B-CLL cells synthesize DNA in response to low molecular weight BCGF, although this response is heterogeneous among different patient samples. IFN- $\alpha$  inhibited BCGF/SAC I-driven proliferation in B-CLL cells in some experiments; however, this was not a consistent finding. In contrast, anti-Leu-13 consistently suppressed BCGF-dependent DNA synthesis in B-CLL cells from early and advanced stage patients. Of particular interest, in leukemic B cells that were not highly responsive to the direct antiproliferative effects of IFN- $\alpha$  in vitro, Leu-13 was upregulated by IFN- $\alpha$

and anti-Leu-13 inhibited BCGF-dependent DNA synthesis. These results indicate that resistance to the growth inhibitory effects of IFN- $\alpha$  in B-cell leukemia can be circumvented via signaling of the IFN-inducible protein Leu-13.

Although these studies do not directly address the potential activation of effector mechanisms including T-cell and natural killer (NK) cell mediated cytotoxicity, the antiproliferative actions of IFN- $\alpha$  and anti-Leu-13 most likely involve a direct effect on leukemic B cells because: (1) hairy cells are relatively resistant to NK cytotoxicity even in the presence of IFN- $\alpha$ <sup>30</sup>; (2) murine IgG<sub>1</sub> antibodies, in contrast to IgG<sub>2a</sub>, do not mediate antibody-dependent cellular cytotoxic activity in human peripheral blood leukocytes (PBL)<sup>31</sup>; and (3) anti-Leu-1, an IgG<sub>2a</sub> $\kappa$ , failed to mediate an antiproliferative effect in B-CLL cultures. Therefore, the capacity of anti-Leu-13 to potentiate the antiproliferative effect of IFN- $\alpha$  on BCGF-stimulated growth of leukemic B cells would appear to occur independently of effector cytotoxicity mechanisms.

The importance of IFN-inducible proteins in mediating the antiproliferative effects of IFN- $\alpha$  has been implicated in several recent studies. Hillman et al<sup>32</sup> have reported that resistance to the growth inhibitory effects of IFN in the Namalva B cell line is associated with the absence of expression of a 17-Kd IFN-inducible cell surface protein. In these studies,<sup>32</sup> IFN resistance could be reversed by the addition of partially purified 17-Kd protein to Namalva cells. Kessler et al<sup>33</sup> have recently examined the pathways involved in the transcriptional regulation of IFN-inducible proteins in IFN-sensitive and resistant B-cell lines. These investigators demonstrated a failure to upregulate specific IFN-inducible proteins in some IFN-resistant B-cell lines that was associated with a defect in the ability of IFN- $\alpha$  to activate DNA-binding factors necessary to trigger IFN-stimulated gene transcription. In the present study, defective upregula-

tion of Leu-13 in response to IFN- $\alpha$  in vitro was observed in only two experiments involving leukemic B cells from early stage CLL patients. In a vast majority of patient samples studied, IFN- $\alpha$  effectively increased the cell surface density of Leu-13 on leukemic B cells. Thus, although the molecular mechanisms involved in the upregulation of Leu-13 are not presently known, our data indicate that resistance to the antiproliferative effects of IFN- $\alpha$  on leukemic B cells in vitro is not generally associated with a defect in the regulation of synthesis of the IFN-inducible protein Leu-13. IFN resistance in B-cell leukemia may therefore occur at another level, possibly through the inappropriate signaling of IFN-inducible cell surface proteins.

Taken together, these studies provide insight into the mechanism of action of IFN- $\alpha$  in leukemic B cells. The data presented support the conclusion that the IFN-inducible protein Leu-13 plays a role in regulating the growth and cellular adhesion properties of leukemic B cells. Moreover, evidence indicating that resistance to the antiproliferative effects of IFN- $\alpha$  can be circumvented via signaling of the IFN-inducible protein Leu-13 may have future therapeutic implications. These initial studies suggest that the combined use of IFN- $\alpha$  and MoAbs to IFN-inducible proteins may represent a new approach for achieving therapeutic effects in B-cell leukemias that are nonresponsive to IFN- $\alpha$  treatment.

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